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(71) Applicant *(for all designated States except US)*: **MCGILL UNIVERSITY** [CA/CA]; 845 Sherbrooke Street West, Montreal, Quebec H3A 2T5 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BRANTON, Philip, E.** [CA/CA]; 266 Macaulay Street, St-Lambert, Quebec J4R 2G9 (CA). **LAI, Albert** [CA/CA]; 35 Wintermute Blvd., Scarborough, Ontario M1W 3M5 (CA).

(74) Agents: **DUBUC, Jean, H.** et al.; Goudreau Gage Dubuc & Martineau Walker, The Stock Exchange Tower, Suite 3400, P.O. Box 242, 800 Place-Victoria, Montreal H4Z 1E9 (CA).

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(54) Title: RBP1 POLYPEPTIDES AND USES THEREOF

(57) Abstract: The invention features methods for modulating cell proliferation, methods for identifying compounds that modulate cell proliferation, and methods for determining whether a patient has a cell proliferation disease. The invention also features pharmaceutical compositions that include RBP1 polypeptides or nucleic acids, and uses thereof.

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RBP1 POLYPEPTIDES AND USES THEREOF

Background of the Invention

The invention relates to the regulation of genc transcription and cell proliferation.

Control of gene expression by the E2F family of transcription factors plays an important role in the cell cycle and growth arrest, as E2F regulates expression of a number of genes required for DNA synthesis and cell cycle progression. E2F appears to be a major target of the retinoblastoma (RB) family of growth suppressors, pRB, p130 and p107, that bind to and inhibit E2F. Interactions between the E2F and RB families are cell cycle-dependent. pRB-E2F complexes are found throughout the cell cycle and in growth-arrested cells, and inhibition of E2F activity appears to be regulated at least in part by phosphorylation of pRB by cyclin-dependent protein kinases (Cdk). p107-E2F complexes are found largely in late G1 and S-phase, and also may regulate cell cycle progression. p130-E2F complexes are prominent in growth-arrested cells; in this context, the complexes have been shown to lack Cdk2 and cyclins.

Summary of the Invention

In a first aspect, the invention features a method for modulating (e.g., decreasing) proliferation of a cell. The method includes contacting the cell with an RBP1 polypeptide that modulates proliferation of the cell. In one preferred embodiment, contacting is achieved by expressing in the cell a nucleic acid molecule encoding the RBP1 polypeptide.

In a second aspect, the invention features another method for modulating (e.g., decreasing) proliferation of a cell. The method includes contacting the cell with a compound that modulates RBP1 biological activity or expression.

In a third aspect, the invention features a method for identifying a

compound that modulates cell proliferation. The method generally includes contacting a cell with a candidate compound and monitoring the level of expression of an RBP1 polypeptide, wherein a change in the level of expression in response to the candidate compound, relative to a level of expression in a cell not contacted with the candidate compound, identifies the compound as a compound that modulates cell proliferation.

In a fourth aspect, the invention features another method for identifying a compound that modulates cell proliferation. The method includes: (a) providing an RBP1 polypeptide having biological activity, (b) contacting the polypeptide with a candidate compound, and (c) monitoring the level of the biological activity of the RBP1 polypeptide, wherein a change in the level of biological activity in response to the candidate compound, relative to a level of biological activity of an RBP1 polypeptide not contacted with the candidate compound, identifies the compound as a compound that modulates cell proliferation. The RBP1 polypeptide can be in a cell or in a cell-free system.

In a fifth aspect, the invention features a method for determining whether a first mammal (e.g., a human patient) has a cell proliferation disease or increased likelihood of developing the disease. The method includes measuring the level of expression of an RBP1 polypeptide in a sample from the mammal, wherein an increase in the level of expression of the RBP1 polypeptide, relative to a matched sample from a second mammal (e.g., an unaffected person) indicates that the first mammal has the cell proliferation disease or increased likelihood of developing the disease. In preferred embodiments, the expression is measured by assaying the amount of RBP1 polypeptide or RBP1 RNA in the sample.

In a sixth aspect, the invention features another method for determining whether a first mammal (e.g., a human patient) has a cell proliferation disease or an increased likelihood of developing the disease. The method includes

measuring the level of RBP1 biological activity in a sample from the mammal, wherein an alteration in the level of the biological activity, relative to a level of RBP1 biological activity in a matched sample from a second mammal (e.g., an unaffected person), indicates that the first mammal has the disease or increased likelihood of developing the disease.

In one preferred embodiments of the invention, the RBP1 biological activity is binding of the RBP1 polypeptide to an HDAC or to a protein (e.g., SAP30, SinA, or SinB) that is part of a protein complex containing an HDAC. Preferably, binding of the RBP1 polypeptide to the HDAC or the protein does not require the presence of an RB family member. In other preferred embodiments, the biological activity is binding of the RBP1 polypeptide to an E2F protein or is repression of E2F-mediated transcription. Preferably, the repression of E2F-mediated transcription does not require the presence of an HDAC protein. In particularly preferred embodiments of the invention, the cell is from a mammal (e.g., a human or a rodent) or in a mammal.

In a seventh aspect, the invention features a method of repressing transcription in a cell. The method includes introducing into the cell a polypeptide having: (i) a repressor domain from RBP1, and (ii) a DNA binding domain. In one preferred embodiment, this is achieved by expressing in the cell a nucleic acid molecule encoding the repressor domain from RBP1 and the DNA binding domain.

In an eighth aspect, the invention features a polypeptide that includes: (i) a repressor domain from RBP1, and (ii) a DNA binding domain. Preferably, the repressor domain includes amino acids 241-452 or amino acids 1167-1257 of SEQ ID NO: 4.

In a ninth aspect, the invention features a nucleic acid molecule encoding a polypeptide having: (i) a repressor domain from RBP1, and (ii) a DNA binding domain.

In a tenth aspect, the invention features a composition that includes a substantially purified RBP1 polypeptide formulated in a physiologically acceptable carrier.

In an eleventh aspect, the invention features composition that includes a substantially purified antibody that specifically binds an RBP1 polypeptide formulated in a physiologically acceptable carrier.

In a twelfth aspect, the invention features a kit for diagnosing a mammal (e.g., a human) with a cell proliferation disease or an increased likelihood of developing the disease. The kit includes a substantially pure antibody that specifically binds an RBP1 polypeptide. Preferably, the kit also includes a means for detecting the binding of the antibody to the RBP1 polypeptide.

By "interact" or "bind" is meant that two polypeptides can transitorily bind, either directly or indirectly, to each other. Indirect binding can take place, for example, through the formation of a multi-protein complex. For example, HDACs are known to be part of a multi-protein complex that also includes, e.g., SAP30, SinA, and SinB (Pazin and Kadonaga, Cell 89:325-328, 1997). The interaction is dependent on inherent properties of the two polypeptides, and specifically excludes non-specific (e.g., strictly Brownian) contact. Preferably, this interaction can be disrupted or prevented by alteration in the primary, secondary, or tertiary structure of one or both of the polypeptides.

By a "transcriptional repressor domain" is meant a polypeptide domain that retains its transcriptional repressor function when the domain is transferred to a heterologous protein. Preferably, the heterologous protein contains nuclear localization and DNA-binding domains.

By "RBP1 biological activity" is meant (i) RBP1 binding to an HDAC protein or a protein complex containing an HDAC, (ii) repression of E2F transcriptional activity, and (iii) arrest or decrease of cell cycle progression.

In addition to RBP1, other, related, polypeptides are highly likely to

have similar biological activities as RBP1, and, thus, are considered to be part of the invention. These polypeptides include ARID domain-containing polypeptides, such as the ones described herein, and the polypeptide fragment of GenBank accession number AAD41239 (PID g5257005).

By "E2F" or "E2F protein" is meant a protein having E2F transcriptional activity. Preferred E2F proteins include E2F-1, E2F-2, E2F-3, E2F-4, and E2F-5.

By "RB family member" is meant the tumor suppressors pRb, p130, and p107.

By an "HDAC" is meant any histone deacetylase protein. Preferred HDACs are HDAC-1, HDAC-2, and HDAC-3.

By a "substantially purified" protein or antibody is meant a protein or antibody that has been separated from components that naturally accompany it. Typically, the protein is substantially purified when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, a polypeptide of the invention. A substantially purified protein of the invention may be obtained, for example, by extraction from a natural source (for example, a virus), by expression of a recombinant nucleic acid encoding such a protein, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly,

substantially purified polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

By "compound" or "candidate compound" is meant a chemical, be it naturally-occurring or artificially-derived, that is assayed for its ability to modulate an alteration in reporter gene activity or protein levels, by employing one of the assay methods described herein. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

By "assaying" is meant analyzing the effect of a treatment, be it chemical or physical, administered to whole animals or cells derived therefrom. The material being analyzed may be an animal, a cell, a lysate or extract derived from a cell, or a molecule derived from a cell. The analysis may be, for example, for the purpose of detecting altered gene expression, altered RNA stability, altered protein stability, altered protein levels, or altered protein biological activity. The means for analyzing may include, for example, antibody labeling, immunoprecipitation, phosphorylation assays; and methods known to those skilled in the art for detecting nucleic acids and polypeptides.

By "modulating" is meant changing, either by decrease or increase, biological activity.

By "a decrease" is meant a lowering in the level of biological activity, as measured by a lowering of (i) RBP1 protein, as measured by ELISA, (ii) RBP1 transcriptional repression of E2F-dependent transcription, as measured, for example, by reporter gene assay (*lacZ*/β-galactosidase, green fluorescent protein, luciferase, etc.), (iii) RBP1 mRNA levels, as measured by PCR relative to an internal control, for example, a "housekeeping" gene product such as β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (iv) the amount of HDACs (or any other protein that is part of a protein complex that contains an HDAC) that interact with RBP1, (v) the

amount of E2F protein that interacts with RBP1, and (vi) RBP1-mediated cell cycle arrest. In all cases, the lowering is preferably by 20%, more preferably by 40%, and even more preferably by 70%.

By an "increase" is meant a rise in the level of biological activity, as measured by an increase of: (i) RBP1 protein, as measured by ELISA, (ii) RBP1 transcriptional repression of E2F-dependent transcription, as measured, for example, by reporter gene assay (*lacZ*/β-galactosidase, green fluorescent protein, luciferase, etc.), (iii) RBP1 mRNA levels, as measured by PCR relative to an internal control, for example, a "housekeeping" gene product such as β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), (iv) the amount of HDACs (or any other protein that is part of a protein complex that contains an HDAC) that interact with RBP1, (v) the amount of E2F protein that interacts with RBP1, and (vi) RBP1-mediated cell cycle arrest. Preferably, the increase is by 5% or more, more preferably by 15% or more, even more preferably by 2-fold, and most preferably by at least 3-fold.

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those regulatory elements that are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific, temporal-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' or intron sequence regions of the native gene.

By "operably linked" is meant that a gene and a promoter are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the promoter.

By "pharmaceutically acceptable" is meant that a carrier is substantially non-toxic to the treated mammal and retains the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier is physiological saline solution. Other physiologically

acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington: The Science and Practice of Pharmacy, (19th ed.) ed. A.R. Gennaro, 1995, Mack Publishing Company, Easton, PA.

By a "matched" sample is meant one derived from the same or similar cells or tissue, from an organism of the same species, to which it is being compared.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof.

Description of the Drawings

Fig. 1 is a photograph of a autoradiograph showing analysis of E2F complexes by EMSA. Components of the E2F complexes were identified by antibody "supershift" experiments as described herein. Lane 1: asynchronously growing cells; lanes 2 to 4: extracts from asynchronously growing cells incubated in the presence anti-pRB serum (lane 2), anti-p107 serum SD9 (lane 3), or anti-p130 serum C-20 (lane 4); lane 5: serum-starved cells; lanes 6 to 8: extracts from serum-starved cells incubated in the presence of anti-pRB (lane 6), anti-p107 (lane 7) or anti-p130 (lane 8) sera; lanes 9 to 12: asynchronously growing cells (lane 9), serum-starved cells (lane 10), or extracts from serum-starved cells incubated in the presence of anti-p130 antibody (lane 11) or anti-RBP1 serum (lane 12); lanes 9A to 12A: the same samples as in lanes 9 to 12 analyzed using a polyacrylamide:bis-acrylamide ratio of 19:1. The positions of p130-E2F, and pRB-containing E2F complexes (RB) and those of "supershifted" complexes (*p130-E2F and *RB) are as indicated.

Figs. 2A and 2B are schematic illustrations showing the effect of RBP1 on E2F-dependent transcription. Human C33A cells were transiently transfected with E2F1-Luc(Wt) (Fig. 2A), which contains firefly luciferase

linked to an E2F-1 specific promoter, or E2F1-Luc(mE2F) (Fig. 2B), which contained mutated E2F recognition sites. They were also co-transfected with increasing amount (from 1 μg - 4 μg) of plasmids pCMVRBP1-HA (human RBP126), pCMVRBP1ΔLCE-HA (a deletion mutant lacking RBP1 residues 921-1056 including the Leu-X-Cys-X-Glu binding site), and, in some cases, plasmids expressing human pRB, p130, or the product of the 12S Ad5 E1A mRNA subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) and expressed under the cytomegalovirus (CMV) promoter. Cells were harvested at 40 hours post-transfection and luciferase activity was measured as previously described (Weintraub et al., Nature 375:812-815, 1995). Results are presented as a percentage of that obtained with reporter plasmid alone. Fig. 2C is a schematic illustration showing the effects of RBP1(Wt) and RBP1(ΔLCE) on pRB and p130 repressed E2F1(Wt) promoter. Cells were transfected as in Fig. 2A. Additional samples were assayed by co-transfecting plasmids expressing pRB or p130 with increasing amount of plasmids pCMVRBP1-HA expressing RBP1(Wt) or pCMVRBP1ΔLCE-HA expressing RBP1(ΔLCE) mutant. Measured luciferase activities are presented as a percentage of that obtained with reporter plasmid alone and are plotted against amount of pCMVRBP1-HA or pCMVRBP1(ΔLCE) plasmids transfected.

Fig. 3A is a schematic illustration showing inhibition of colony formation by RBP1. CV-1 cells were transfected with plasmids expressing neo and either wild-type RBP1 or a truncated form of RBP1 that lacks the LXCXE-RB binding motif. Cells were grown under drug selection for two weeks. Colonies were then counted and the calculated averages were compared with the number of colonies obtained with cells expressing neo alone (pcDNA3). Fig. 3B is a series of photographs showing morphology of colonies resulting from the experiment described in Fig. 3A.

Fig. 4A is a schematic illustration showing repression of Gal4-dependent

transcription by RBP1. Fig. 4B is a schematic illustration showing RBP1 deletion mutants and a summary of CAT activity as a percent of control.

Figs. 5A and 5B are a series of photographs showing interactions of pRB and p107 with histone deacetylases. The locations of hypophosphorylated species of pRB and p107 are as indicated.

Figs. 6A - 6D show *in vivo* interaction of the small 'pocket' of pRB with histone deacetylases. cDNAs encoding Gal4DBD fused with the 'pocket' of pRB and those encoding Flag-tagged HDAC1, HDAC2, or HDAC3 were overexpressed in H1299 (Fig. 6A). Anti-Gal4DBD antibody was used to immunoprecipitate Gal4-tagged overexpressed proteins and co-immunoprecipitation of HDACs was then detected by western blotting. WC - whole cell extracts; IP - anti-Gal4 immunoprecipitated proteins. Binding studies similar to those described in Fig. 6A were also performed in 293T cells (Fig. 6B) and 293 cells (Fig. 6C). A modified binding experiment similar to that described above was performed in H1299 cells by incubating cell extracts with increasing amounts (0 to 20 μg) of E1A protein (first exon containing the LXCXE motif) fused to GST (GST-E1A) (Fig. 6D). The positions of migration of Flag-tagged HDAC1 (open arrows), HDAC2 (closed arrow) and HDAC3 (arrowhead) are as indicated.

Figs. 7A - 7E are a series of photographs showing *in vivo* interaction of RBP1 with histone deacetylases. Binding studies similar to those described in Fig. 6 were performed in H1299 cells (Fig. 7A) or 293T cells (Fig. 7B) using Gal4-RBP1 instead of Gal4-pRB. Binding studies were also performed in 293T cells transfected with the RBP1-HA construct and Gal4-HDAC1, Gal4-HDAC2, and Gal4-HDAC3 constructs (Fig. 7C). Coimmunoprecipitation studies similar were done in both H1299 and 293T cells. In addition to the antisera described, antibody against pRB, p130, p107, and the HA-epitope were also used (Fig. 7D). A binding study was also done using the Gal4-RBP1-

ΔLCE and RBP1-ΔLCE-HA mutants, each of which lacks the LXCXE 'pocket'-binding motif (Fig. 7E). The positions of migration of Flag-tagged HDAC1 (open arrows), HDAC2 (closed arrow) and HDAC3 (arrowhead) are as indicated.

Figs. 8A - 8D are a series of schematic illustrations showing mapping of transcriptional repression domains in RBP1 and the effect of trichostatin A (TSA) on RBP1 repression activity. CAT assays were performed in CHO cells using G5TKCAT as reporter. A summary of repression results obtained in experiments described in Fig. 8A are shown at the left of Fig. 8B. The effect of TSA on repression is shown in Fig. 8C. Repression assays were carried out as in Fig. 8A, except that some cells were incubated with 330 nM TSA for 24 hours prior to harvesting and the G5MLPCAT reporter was assayed instead of the G5TKCAT reporter. Fig. 8D shows a similar experiment to that described in Fig. 8C, except performed using a reporter construct consisting of the E2F-1 promoter linked to a cDNA encoding luciferase and either RBP1, RBP1-ΔLCE, the 'pocket' of pRB, or an inactive pRB 'pocket'- mRB(C706F) mutant.

Fig. 9 is a schematic illustration showing mapping of specific binding of HDAC3 to RBP1. Binding studies were performed in 293T cells using either Gal4-R1, Gal4-R2(1314-C)/(1263-C), Gal4 alone, Gal4-RBP1(wt), Gal4-ARID, Gal4-ΔR1, or Gal4-Δ93C(Δ-R2).

Detailed Description of the Invention

We have discovered that RBP1, a known pRB pocket-binding protein, possesses transcriptional repression activity and associates with p130-E2F and pRB-E2F complexes specifically during growth arrest. Overexpression of RBP1 both inhibited E2F-dependent gene expression and suppressed cell growth. Thus, we believe that repression of E2F-dependent transcription by RBP1 via RB family members plays a central role in inducing growth arrest.

We have also discovered that RBP1 interacts with histone deacetylases (HDACs). Unlike pRB-histone deacetylase interactions, however, 'pocket'-binding E1A protein and large T antigen do not affect interactions between RBP1 and any of the histone deacetylases. It was previously thought that HDAC1 or HDAC2 interacted directly with the RB pocket via degenerate IXCXE motifs, but this model does not account for interactions of HDAC3, which lacks such sequence. Our data indicated that binding of HDAC3 to RB family members also requires the pocket, suggesting that such interactions may be indirect and rely on a pocket-binding protein, such as RBP1. Our results show RBP1's functional importance in the RB family-mediated repression of E2F-dependent transcription, by linking histone deacetylases to the pockets of RB family proteins.

We have also identified two repressor domains in RBP1. One of these domains is HDAC-dependent, while the other is HDAC-independent.

Thus, we have found for the first time that RBP1 is a key member of the RB-E2F complex, serving in part as a link between RB and HDACs, but also serving a second, HDAC-independent, function. This discovery allows for the development of drugs that modulate or mimic RBP1 biological activity or that modulate its expression. These drugs have use in the treatment of disorders characterized by abnormal proliferation or cell death.

Production of RBP1 polypeptide

For protein expression, eukaryotic and prokaryotic expression systems may be generated in which *RBP1* gene sequences are introduced into a plasmid or other vector, which is then used to transform living cells. Constructs in which the RBP1 cDNAs containing the entire open reading frames inserted in the correct orientation into an expression plasmid may be used for protein expression. Alternatively, portions of the *RBP1* gene sequences, including

wild-type or mutant *RBP1* sequences, may be inserted. Prokaryotic and eukaryotic expression systems allow various important functional domains of the RBP1 proteins to be recovered as fusion proteins and then used for binding, structural and functional studies and also for the generation of appropriate antibodies. Since RBP1 protein expression may induce cell cycle arrest in some cell types, it may be desirable to express the protein under the control of an inducible promoter.

Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the inserted *RBP1* nucleic acid in the plasmid bearing cells. They may also include eukaryotic or prokaryotic origin of replication sequences allowing for their autonomous replication within the host organism, sequences that encode genetic traits that allow vector-containing cells to be selected for in the presence of otherwise toxic drugs, and sequences that increase the efficiency with which the synthesized mRNA is translated. Stable long-term vectors may be maintained as freely replicating entities by using regulatory elements of, for example, viruses (e.g., the OriP sequences from the Epstein Barr Virus genome). Cell lines may also be produced which have integrated the vector into the genomic DNA, and in this manner the gene product is produced on a continuous basis.

Expression of foreign sequences in bacteria, such as *Escherichia coli,* requires the insertion of the *RBP1* nucleic acid sequence into a bacterial expression vector. This plasmid vector contains several elements required for the propagation of the plasmid in bacteria, and expression of inserted DNA of the plasmid by the plasmid-carrying bacteria. Propagation of only plasmid-bearing bacteria is achieved by introducing in the plasmid selectable marker-encoding sequences that allow plasmid-bearing bacteria to grow in the presence of otherwise toxic drugs. The plasmid also bears a transcriptional promoter capable of producing large amounts of mRNA from the cloned gene. Such

promoters may or may not be inducible promoters, which initiate transcription upon induction. The plasmid also preferably contains a polylinker to simplify insertion of the gene in the correct orientation within the vector. In a simple *E. coli* expression vector utilizing the lac promoter, the expression vector plasmid contains a fragment of the *E. coli* chromosome containing the lac promoter and the neighboring *lacZ* gene. In the presence of the lactose analog IPTG, RNA polymerase normally transcribes the *lacZ* gene, producing *lacZ* mRNA, which is translated into the encoded protein, β-galactosidase. The *lacZ* gene can be cut out of the expression vector with restriction endonucleases and replaced by a *RBP1* gene sequence, or fragment, fusion, or mutant thereof. When this resulting plasmid is transfected into *E. coli*, addition of IPTG and subsequent transcription from the lac promoter produces *RBP1* mRNA, which is translated into a RBP1 polypeptide.

Once the appropriate expression vectors containing a *RBP1* gene, or fragment, fusion, or mutant thereof, are constructed, they are introduced into an appropriate host cell by transformation techniques including, for example, calcium phosphate transfection, DEAE-dextran transfection, electroporation, microinjection, protoplast fusion, and liposome-mediated transfection, as described herein. The host cells that are transfected with the vectors of this invention may include (but are not limited to) *E. coli*, *Pseudomonas*, *Bacillus subtilus*, or other bacilli, other bacteria, yeast, fungi, insect (using, for example, baculoviral vectors for expression), mouse or other animal or human tissue cells. Mammalian cells can also be used to express the RBP1 protein using a vaccinia virus expression system described in Ausubel et al., 1997, Current Protocols in Molecular Biology, Wiley Interscience, New York.

In vitro expression of RBP1 proteins, fusions, polypeptide fragments, or mutants encoded by cloned DNA is also possible using the T7 late-promoter expression system. This system depends on the regulated expression of T7

RNA polymerase which is an enzyme encoded in the DNA of bacteriophage T7. The T7 RNA polymerase transcribes DNA beginning within a specific 23-bp promoter sequence called the T7 late promoter. Copies of the T7 late promoter are located at several sites on the T7 genome, but none is present in *E. coli* chromosomal DNA. As a result, in T7 infected cells, T7 RNA polymerase catalyzes transcription of viral genes but not of *E. coli* genes. In this expression system recombinant *E. coli* cells are first engineered to carry the gene encoding T7 RNA polymerase next to the lac promoter. In the presence of IPTG, these cells transcribe the T7 polymerase gene at a high rate and synthesize abundant amounts of T7 RNA polymerase. These cells are then transformed with plasmid vectors that carry a copy of the T7 late promoter protein. When IPTG is added to the culture medium containing these transformed *E. coli* cells, large amounts of T7 RNA polymerase are produced. The polymerase then binds to the T7 late promoter on the plasmid expression vectors, catalyzing transcription of the inserted cDNA at a high rate. Since each *E. coli* cell contains many copies of the expression vector, large amounts of mRNA corresponding to the cloned cDNA can be produced in this system and the resulting protein can be radioactively labeled. Plasmid vectors containing late promoters and the corresponding RNA polymerases from related bacteriophages such as T3, T5, and SP6 may also be used for *in vitro* production of proteins from cloned DNA. *E. coli* can also be used for expression using an M13 phage such as mGPI-2. Furthermore, vectors that contain phage lambda regulatory sequences, or vectors that direct the expression of fusion proteins, for example, a maltose-binding protein fusion protein or a glutathione-S-transferase fusion protein, also may be used for expression in *E. coli.*

Eukaryotic expression systems permit appropriate post-translational modifications to expressed proteins. Transient transfection of a eukaryotic expression plasmid allows the transient production of a RBP1 polypeptide by a

transfected host cell. RBP1 proteins may also be produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public (e.g., see Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, Supp. 1987 and VectorDB at URL address http://vectordb.atcg.com/), as are methods for constructing such cell lines (see e.g., Ausubel et al., *supra*). In one example, cDNA encoding a RBP1 protein, fusion, mutant, or polypeptide fragment is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, integration of the RBP1-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (as described, Ausubel et al., *supra*). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al., *supra*. These methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. The most commonly used DHFR-containing expression vectors are pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., *supra*). The host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR$^-$ cells, ATCC Accession No. CRL 9096) are among those most preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

Eukaryotic cell expression of RBP1 proteins allows the production of large amounts of normal and mutant proteins for isolation and purification, and the use of cells expressing RBP1 proteins as a functional assay system for antibodies generated against the protein. Eukaryotic cells expressing RBP1 proteins may also be used to test the effectiveness of pharmacological agents on RBP1 biological activity, or as means by which to study RBP1 proteins as

components of a transcriptional regulatory complex. Expression of RBP1 proteins, fusions, mutants, and polypeptide fragments in eukaryotic cells also enables the study of the function of the normal complete protein, specific portions of the protein, or of naturally occurring polymorphisms and artificially produced mutated proteins. The *RBP1* DNA sequences can be altered using procedures known in the art, such as restriction endonuclease digestion, DNA polymerase fill-in, exonuclease deletion, terminal deoxynucleotide transferase extension, ligation of synthetic or cloned DNA sequences and site-directed sequence alteration using specific oligonucleotides together with PCR.

Another preferred eukaryotic expression system is the baculovirus system using, for example, the vector pBacPAK9, which is available from Clontech (Palo Alto, CA). If desired, this system may be used in conjunction with other protein expression techniques, for example, the myc tag approach described by Evan et al. (*Mol. Cell Biol.* 5:3610-3616, 1985).

Once the recombinant protein is expressed, it can be isolated from the expressing cells by cell lysis followed by protein purification techniques, such as affinity chromatography. In this example, an anti-RBP1 antibody can be attached to a column and used to isolate the recombinant RBP1 proteins. Lysis and fractionation of RBP1 protein-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., *supra*). Once isolated, the recombinant protein can, if desired, be purified further by e.g., by high performance liquid chromatography (HPLC; e.g., see Fisher, Laboratory Techniques In Biochemistry And Molecular Biology, Work and Burdon, Eds., Elsevier, 1980).

Polypeptides of the invention, particularly short RBP1 fragments and longer fragments of the N-terminus and C-terminus of the RBP1 protein, can also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984, The Pierce Chemical Co., Rockford,

IL). These general techniques of polypeptide expression and purification can also be used to produce and isolate useful RBP1 polypeptide fragments or analogs, as described herein.

Those skilled in the art of molecular biology will understand that a wide variety of expression systems may be used to produce the recombinant RBP1 proteins. The precise host cell used is not critical to the invention. The RBP1 proteins may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *S. cerevisiae*, insect cells such as Sf9 cells, or mammalian cells such as COS-1, NIH 3T3, or HeLa cells). These cells are commercially available from, for example, the American Type Culture Collection, Rockville, MD (see also F. Ausubel et al., *supra*). The method of transformation and the choice of expression vehicle (e.g., expression vector) will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al., *supra*, and expression vehicles may be chosen from those provided, e.g., in Pouwels et al., *supra*.

Testing for the presence of RBP1 biological activity

Identification of both biologically active and mutant forms of RBP1 allows the study of RBP1 biological activity in regulation of cell proliferation. For example, administration of an RBP1 protein, or a polypeptide fragment thereof, can be used to inhibit E2F-regulated gene expression, as measured by cell-based and cell-free assays known in the art and described herein. An inhibitory amount of an RBP1 reagent (e.g., a compound that reduces the biological function of RBP1, and thus may promote cell proliferation, such as an RBP1 neutralizing antibody or antisense *RBP1* nucleic acid, an *RBP1* nucleic acid encoding a dominant-negative form of the RBP1 protein, or a compound which decreases RBP1 gene expression) may be similarly assessed. Such assays may be carried out in a cell which either expresses endogenous

RBP1 or a cell to which is introduced a heterologous amount of an RBP1 polypeptide or in a cell-free assay. Preferably, the cell is capable of undergoing RBP1-mediated cell cycle arrest. RBP1 biological activity or inhibition thereof may be assessed in these RBP1 expressing cells, whereby such RBP1-inducing or -inhibiting activity is evaluated based upon the level of RBP1 biological activity.

One preferred RBP1 biological activity is binding of RBP1 to an HDAC. It is understood that the binding may not be through direct contact between RBP1 and the HDAC, but may require additional proteins. Binding of RBP1 to an HDAC, or any other protein that is part of a protein complex that contains an HDAC (e.g., SAP30, SinA, or SinB) can be measured using any of a number of standard methods known to those in the art, such as the methods described herein.

Another preferred RBP1 biological activity is binding of RBP1 to an E2F protein. As described for the RBP1-HDAC interaction, the binding of RBP1 to an E2F protein may require additional proteins.

A related RBP1 biological activity is repression of E2F transcriptional activity by RBP1. It is likely that the repression domains of RBP1 are sites in which proteins bind to RBP1 and repress E2F transcriptional activity. E2F transcriptional activity can be measured, for example, using the methods described herein.

In another embodiment, RBP1 biological activity is slowing of the cell cycle or cell cycle arrest. There are numerous cell cycle assays known in the art, including those described herein; any of these may be used in measuring RBP1-mediated cell cycle regulation.

Identification of molecules that modulate RBP1 biological activity

Methods of observing changes in RBP1 biological activity, such as the

ones described above, are exploited in high-throughput assays for the purpose of identifying compounds that modulate mutant or wild-type RBP1 transcriptional activity. Compounds that mime RBP1 activity also may be identified by such assays. Furthermore, compounds that modulate transcription of the *RBP1* gene itself may be identified; in some cases, it may be desirable to increase or decrease RBP1 protein levels by such an approach. Such identified compounds may have utility as therapeutic agents in the treatment of cell proliferation disease.

In general, novel drugs that modulate or mime RBP1 biological activity or expression are identified from large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge,

MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their therapeutic activities for cell proliferation diseases should be employed whenever possible.

When a crude extract is found to modulate RBP1 biological activity, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract that modulates or mimes RBP1 biological activity or expression. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed using a standard animal or culture model for a cell proliferative disease known in the art.

The effect of candidate compounds on RBP1-mediated regulation of cell growth or survival may be measured at the level of translation by using the general approach described above with standard protein detection techniques, such as Western blotting or immunoprecipitation with an RBP1-specific

antibody (for example, the RBP1-specific antibody described herein).

Compounds that modulate the level of RBP1 may be purified, or substantially purified, or may be one component of a mixture of compounds such as an extract or supernatant obtained from cells (Ausubel et al., *supra*). In an assay of a mixture of compounds, RBP1 expression is measured in cells administered progressively smaller subsets of the compound pool (e.g., produced by standard purification techniques such as HPLC or FPLC) until a single compound or minimal number of effective compounds is demonstrated to modulate RBP1 expression.

Compounds may also be screened for their ability to modulate RBP1 biological activity. In this approach, the degree of RBP1 biological activity in the presence of a candidate compound is compared to the degree of biological activity in its absence, under equivalent conditions. Again, the screen may begin with a pool of candidate compounds, from which one or more useful modulator compounds are isolated in a step-wise fashion. Biological activity may be measured by any standard assay, for example, those described herein.

Another method for detecting compounds that modulate the activity of RBP1 is to screen for compounds that bind to RBP1. These compounds may be detected by adapting interaction trap expression systems known in the art. These systems detect protein interactions using a transcriptional activation assay and are generally described by Gyuris et al. (Cell 75:791-803, 1993) and Field et al., (Nature 340:245-246, 1989), and are commercially available from Clontech (Palo Alto, CA). Alternatively, compounds that bind to RBP1 can be isolated by column chromatography. In this assay, candidate compounds or extracts are passed through a column containing immobilized RBP1. After washing to remove nonspecific proteins, the compounds bound to RBP1 are eluted, collected, and identified. Using similar assays, compounds that interfere with RBP1 binding to pRB family members or HDACs can also be

identified.

Compounds or molecules that modulate RBP1 biological activity may include peptide and non-peptide molecules such as those present in cell extracts, mammalian serum, or growth medium in which mammalian cells have been cultured.

Therapeutic uses

A molecule that decreases RBP1 expression or biological activity is considered useful in the invention; such a molecule may be used, for example, as a therapeutic to increase cell proliferation in an arrested cell (e.g., a neuron). There is a tight association between cell proliferation and cell death. In some cases, a cell that receives a signal to proliferate will instead undergo apoptosis, presumably because the proliferation is inappropriate. Hence, depending on the context, a molecule that decreases RBP1 expression or biological activity may result in increased proliferation or, alternatively, cell death. Differentiating between these two outcomes in the appropriate context may readily be done using standard assays known to those skilled in the art.

A molecule that increases RBP1 expression or biological activity is particularly useful in the invention. Such a molecule may be used to decrease cellular proliferation. This would be advantageous in the treatment of neoplasms or other cell proliferative diseases.

To add an RBP1 polypeptide to cells in order to modulate cell proliferation or apoptosis, it is preferable to obtain pure RBP1 protein from cultured cell systems that can express the protein. Delivery of the protein to the affected tissue can then be accomplished using appropriate packaging or administrating systems. Alternatively, small molecule analogs may be used and administered to act as RBP1 agonists or antagonists and in this manner produce a desired physiological effect. Methods for finding such molecules are

provided herein.

Gene therapy is another potential therapeutic approach in which normal copies of the RBP1 gene or nucleic acid encoding RBP1 sense or antisense RNA is introduced into cells. The gene must be delivered to those cells in a form in which it can be taken up and encode for sufficient protein to provide effective function.

Transducing retroviral, adenoviral, and human immunodeficiency viral (HIV) vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression (see, for example, Cayouette and Gravel, Hum. Gene Ther., 8:423-430, 1997; Kido et al. Curr. Eye Res., 15:833-844, 1996; Bloomer et al., J. Virol., 71:6641-6649, 1997; Naldini et al., Science 272:263-267, 1996; Miyoshi et al., Proc. Natl. Acad. Sci. USA, 94:10319-10323, 1997). For example, the full length RBP1 nucleic acid, or portions thereof, can be cloned into a retroviral vector and driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest (such as neurons). Other viral vectors which can be used include adenovirus, adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpes virus, such as Epstein-Barr Virus.

Gene transfer could also be achieved using non-viral means requiring infection *in vitro*. This would include calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes may also be potentially beneficial for delivery of DNA into a cell. Although these methods are available, many of these are of lower efficiency.

Transplantation of normal genes into the affected cells of a patient can also be useful therapy. In this procedure, a normal RBP1 gene is transferred into a cultivatable cell type, either exogenously or endogenously to the patient. These cells are then injected into the targeted tissue(s).

In the constructs described, RBP1 cDNA expression can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in tumor cells may be used to direct RBP1 expression. The enhancers used could include, without limitation, those that are characterized as tissue- or cell-specific in their expression. Alternatively, if a RBP1 genomic clone is used as a therapeutic construct (for example, following isolation by hybridization with the RBP1 cDNA described above), regulation may be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

Antisense based strategies may be employed to explore RBP1 gene function and as a basis for therapeutic drug design. The principle is based on the hypothesis that sequence-specific suppression of gene expression can be achieved by intracellular hybridization between mRNA and a complementary antisense species. The formation of a hybrid RNA duplex may then interfere with the processing/transport/translation and/or stability of the target RBP1 mRNA. Antisense strategies may use a variety of approaches including the use of antisense oligonucleotides and injection of antisense RNA. Antisense effects can be induced by control (sense) sequences, however, the extent of phenotypic changes are highly variable. Phenotypic effects induced by antisense effects are based on changes in criteria such as protein levels, protein activity measurement, and target mRNA levels.

For example, RBP1 gene therapy may also be accomplished by direct administration of antisense *RBP1* mRNA to a cell in which proliferation is desired. The antisense *RBP1* mRNA may be produced and isolated by any standard technique, but is most readily produced by *in vitro* transcription using

an antisense *RBP1* cDNA under the control of a high efficiency promoter (e.g., the T7 promoter). Administration of antisense *RBP1* mRNA to cells can be carried out by any of the methods for direct nucleic acid administration described above.

An alternative strategy for inhibiting RBP1 function using gene therapy involves intracellular expression of an anti-RBP1 antibody or a portion of an anti-RBP1 antibody. For example, a nucleic acid (or fragment thereof) encoding a monoclonal antibody that specifically binds to RBP1 and inhibits its biological activity may be placed under the transcriptional control of a cell type-specific gene regulatory sequence.

Another therapeutic approach within the invention involves administration of a recombinant RBP1 polypeptide (e.g, the one described herein), either directly to the site of a potential or actual cell proliferation event (for example, by injection) or systemically (for example, by any conventional recombinant protein administration technique). The dosage of RBP1 depends on a number of factors, including the size and health of the individual patient, but, generally, between 0.1 mg and 100 mg inclusive are administered per day to an adult in any pharmaceutically acceptable formulation.

Administration of RBP1 Polypeptides, *RBP1* Genes, or Modulators of RBP1 Synthesis or Function

An RBP1 protein, gene, or modulator of RBP1 may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer activating RBP1 antibodies or compounds that activate or mimic RBP1 biological activity to patients suffering from a cell proliferation disease. Administration may begin before the patient is symptomatic. Methods well known in the art for making

formulations are found, for example, in Remington: The Science and Practice of Pharmacy (*supra*). Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for RBP1 modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

Gene Regulation

The transcriptional repressor domains described herein can be used to repress expression of any gene by targeting them to the regulatory region of that gene; the presence of the repressor domain will block transcriptional activation (Badiani et al., Genes Dev. 8:770-782, 1994; Conlon et al., Development 122:2427-2435, 1996). Targeting of the repressor domain can be achieved by producing a fusion protein containing the repressor domain fused in frame to a DNA binding domain known to bind to the desired gene regulatory region. The fusion protein can be produced using standard methods and administered in a pharmaceutically-acceptable carrier to the cell or tissue of interest as described herein for the RBP1 polypeptides. Alternatively, the nucleic acid sequence encoding the fusion protein can be used in gene therapy, as described above.

The following examples are to illustrate the invention. They are not

meant to limit the invention in any way.

Example 1: RBP1 is present in E2F-RB family complexes

Because of its slow migration in E2F-specific electrophoretic mobility shift assays (EMSA), we hypothesized that the p130-E2F complex appeared to contain one or more additional proteins and that such proteins interact with p130 via a site in the RB pocket similar to that utilized by the adenovirus E1A protein which binds via a conserved Leu-X-Cys-X-Glu motif found in several other viral and cellular RB-binding proteins.

To determine if RBP1 was present in E2F complexes, we harvested monkey CV-1 cells from either rapidly growing or serum-starved growth-arrested cultures, and subjected cell extracts to electrophoretic mobility shift assays (EMSA) using an E2F-specific radioactive oligonucleotide, as described previously (Corbeil et al., Oncogene 15:657-668, 1997). Fig. 1 shows that serum-starved (lanes 5 and 10) and asynchronously growing cells (lanes 1 and 9) contain a variety of E2F complexes; we observed the large p130-E2F complex only in the former. No complexes were detected using an unrelated or altered E2F oligonucleotide. The components of these E2F complexes were partially resolved through the addition of antibodies that recognize various RB family members. Addition of anti-pRB serum caused a supershift of at least two faster migrating pRB-E2F complexes (termed RB in the figure) to slower migrating species (*RB) in both growing (lane 2) and serum-starved (lane 6) cells. We did not observe any effect on the migration of p130-E2F with this serum (lane 6). Addition of anti-p107 antibody SD9 had little effect on any of the complexes (lanes 3 and 7); addition of C-20 serum specific for the carboxyl terminus of p130, however, caused a supershift of p130-E2F in extracts from growth-arrested cells (lanes 8 and 11). Anti-p130 serum also made evident the existence of more minor, faster-migrating

p130-E2F complexes that were evident in lane 11, upon prolonged exposure of the gel. To determine if RBP1 is present in p130-E2F or any of the other E2F complexes, we conducted a similar experiment in asynchronously growing and serum-starved CV-1 cells using antiserum against human RBP1. Fig. 1 (lane 12) shows that both p130-E2F and the faster-migrating pRB-E2F complexes, identified in lane 2, had disappeared and that one or more new complexes (termed *RB in the figure) were evident. In addition, analysis of these same samples under different conditions of PAGE indicated the presence of an additional, very slowly-migrating E2F complex (termed p130-E2F in Fig. 1) that just barely entered the gel (lane 12A). Thus it appeared that p130-E2F and certain pRB-E2F complexes in growth arrested cells contain RBP1.

Example 2: RBP1 inhibits E2F-mediated transcription

To analyze the role of RBP1 in RB-E2F complexes, we analyzed rapidly growing C33A human cervical carcinoma cells, which are deficient both for functional p53 and pRB, for their ability to regulate the E2F-1 promoter. We chose this promoter because it contains E2F recognition sites that are tightly regulated during growth arrest by E2F-RB family complexes, particularly p130/E2F (Hsiao et al., Genes Dev. 8:1526-1537, 1994; Johnson et al., Nature 365:349-352, 1993; Johnson et al., Genes Dev. 8, 1514-1525, 1994; Johnson, Oncogene 11:1685-1692, 1995). We transfected cells with DNA from E2F1-Luc(Wt), a luciferase reporter construct driven by the E2F-1 promoter, together with pCMVRBP1-HA plasmid that encodes a haemagglutinin (HA) epitope-tagged RBP1, or pCMVRBP1ΔLCE-HA (a mutant RBP1 lacking the Leu-X-Cys-X-Glu p130/pRB binding site). In some cases, we also assayed DNA from plasmids encoding pRB, p130, or E1A-243R (the 243-residue product of human adenovirus type 5 12S E1A mRNA). As shown in Fig. 2A, increasing the amount of cotransfected RBP1(Wt) resulted in decreasing

amount of luciferase activity. Thus, expression is comparable to the transcriptional repression effect that p130 and pRB each exerted on this E2F1-Luc(wt) reporter. Overexpression of RBP1 caused about a 50% reduction in luciferase activity, whereas introduction of the ΔLCE mutant had no inhibitory effect. Expression of E1A-243R increased luciferase activity by almost nineteen-fold, as was expected. To determine if RBP1-mediated transcriptional repression was specific to the E2F-regulated transcription on this promoter, we used an E2F1-luc(mE2F) reporter, which contains four point mutations within the E2F recognition sites (Johnson, Oncogene 11:1685-1692, 1995). This mutated sequence was unable to interact with E2F in EMSA (Johnson et al., Genes Dev. 8, 1514-1525, 1994). As shown in Fig. 2B, the relative luciferase activity of this reporter was about three- to four-fold higher than the E2F1-luc(wt) reporter. Overexpression of either RBP1(Wt) or RBP1(ΔLCE) had no effect on the mutated E2F1 promoter, suggesting the transcriptional repression by RBP1 is specific to the E2F element within the E2F1 promoter. Taken with the finding that RBP1 and pRB are together in a complex, these results suggest that RBP1 inhibits E2F-dependent transcription and that such inhibition requires binding to RB family members. We obtained similar results with other cell types, including CHO C33A cells, which lack functional pRB. Thus, endogenous p130 and/or p107 must be sufficient for this inhibition.

Overexpression of either p130 or pRB has no effect on the E2F1-luc(mE2F) promoter, whereas overexpression of E1A-243R results in about three-fold activation of this promoter (Fig. 2B). E1A has been shown to enhance Sp1 driven transcription (Weintraub and Dean, Nature 358:259-261, 1992). Additionally, both the wild-type and mutated E2F-1 promoters contain a series of Sp1 recognition sites. These data explain E1A-enhanced transcription of both promoters, as shown in Figs. 2A and 2B. The fact that the

E2F1-luc(Wt) promoter was activated by E1A stronger than the E2F1-luc(mE2F) promoter, suggests that the E2F element within the E2F1 promoter is also effected by E1A overexpression.

Example 3: RBP1 inhibition of E2F-mediated transcription is dependent on interaction with RB family members

We predicted that overexpression of RBP1, together with RB family members, should cause further inhibition to the E2F-1 promoter. This was observed, as shown in Fig. 2C. With increasing amount of RBP1(Wt), along with constant amount of cotransfected p130 or pRB, we detected further inhibition of luciferase activity. Interestingly, with increasing amount of RBP1(ΔLCE), along with constant amount of cotransfected p130 or pRB, we observed a gradual increase of luciferase activity. Our observation of de-repression by the RBP1(ΔLCE) mutant suggests one or more common factor(s), required for RBP1 transcriptional repression effect on the E2F1 promoter, interact with both endogenous RBP1 and transfected RBP1(ΔLCE). Overexpression of RBP1(ΔLCE) would sequester these essential factors from the E2F-1 promoter due to the inability of ΔLCE mutant to interact with the RB family members. The inhibitory effect on E2F-1 promoter by RBP1 required the interaction with RB family members through the LXCXE motif. Accordingly RBP1(ΔLCE) is likely to act as a dominant negative mutant, partially abolishing the transcriptional repression effect of exogenously transfected p130 or pRB on the E2F-1 promoter (Fig. 2C). The fact that RBP1(ΔLCE) could only partially abolish the transcriptional repression effect exerted by the cotransfected p130 or pRB, suggests that pRB or p130 repress transcription by other mechanisms, possibly through interaction with other LXCXE-containing cellular proteins.

Example 4: Overexpression of RBP1 induces growth arrest

As the E2F-p107 complex containing RBP1 is only formed during growth arrest, it seemed possible that RBP1 might play a role in the control of cell proliferation by inhibiting E2F-dependent transcription, thus inducing, enhancing, or maintaining exit from the cell cycle. To determine if RBP1 suppressed cell growth, we transfected CV-1 cells with DNA from plasmids that provide neomycin resistance and express RBP1. Constructs included pRcCMVRBP1-HA (encoding an HA-tagged version of RBP1), pcDNA3Gal4-RBP1 (containing RBP1 fused to the Gal4 DNA-binding motif); and pcDNA3Gal4-RBP1ΔLCE. Following transfection, we plated cells and allowed them to grow under G418 selection. Fig. 3A shows that no colonies were formed in the absence of vectors. With pcDNA3Gal4-RBP1ΔLCE, a significant number of large colonies were produced with a morphology similar to those obtained with the control pcDNA3, lacking RBP1 coding sequences (Fig. 3B). With each of the plasmids expressing wild-type RBP1, we observed a significant reduction in colony formation. In addition, the colonies of cells transfected wild-type RBP1 were considerably smaller and composed of only small numbers of sparse cells (see Fig. 3B).

To examine the mechanism by which RBP1 inhibits E2F activity, we fused the GAL4 DNA binding sequence to RBP1. We then co-transfected CHO cells were with DNA encoding GAL4-RBP1 (or control proteins) and G5TKCAT, a reporter construct that expresses CAT by GAL4-dependent transcription. Fig. 4A shows that GAL4-RBP1, expressed from either the CMV or SV40 promoter, repressed transcription by 80 to 90%. This expression was as great as that by the adenovirus E1B-55kDa product (Gal4AdE1B-55K), known to be a potent transcriptional repressor when linked to the Gal4 DNA binding domain. RBP1-HA that was not linked to Gal4 (pRcCMVRBP1-HA) had no effect on expression of CAT. Thus, RBP1

appears to function as a transcriptional repressor, but only when tethered to a promoter. GAL4-RBP1ΔLCE also exhibited similar repression activity, indicating that repression by RBP1 is independent of RB family binding when fused to a heterologous DNA binding domain.

To identify the region of RBP1 required for repression, we created a series of mutants in the GAL4-RBP1 construct in which increasing regions from the carboxyl terminus of RBP1 were deleted (see Fig 4B). Fig. 4 shows that elimination of sequences encoding up to 805 residues from the carboxyl terminus resulted in little or no reduction of repression activity; removal of additional sequences, however, greatly reduced or eliminated repression. It is likely that residues 241 to 452 are responsible for RBP1 repression activity. This portion of RBP1 contains a sequence predicted to encode an α-helical domain, preceded by a region, termed ARID, found in several other proteins, including the product of dead ringer (dri) from Drosophila melanogaster (Gregory et al., Mol. Cell. Biol. 16:792-799, 1996), SWI1/ADR6 from yeast (Coté et al., Science 265:53-60, 1994), B Cell Regulator of IgH Transcription from mouse (Herrscher et al., Genes Dev. 9:3067-3082, 1995), human Mrf1 and Mrf2A (Huang et al., Nucl. Acids Res. 24:1695-1701, 1996), and RBP2 (Defeo-Jones et al., Nature 353:251-254, 1991; Fattaey et al., Oncogene 8:3149-3156,1993; Kaelin et al., Cell 70: 351-364, 1992; Kim et al., Mol. Cell. Biol. 14: 7256-7264, 1994; Otterson et al., Oncogene 8:949-957, 1993). When we fused residues 241 to 452 of RBP1 to Gal4 (Gal4-RBP1-R1), the resulting fusion protein repressed transcription at a level comparable to wild type Gal4-RBP1 (Fig. 4A), suggesting that this region is responsible for the transcriptional repression activity.

Example 5: Endogenous interactions between RB family members and histone deacetylases

We conducted co-immunoprecipitation experiments to determine the ability of pRB to interact with various HDAC enzymes *in vivo*. Rabbit polyclonal antibodies that specifically recognize HDAC2, or HDAC1 and HDAC3 were used under low stringency conditions to immunoprecipitate endogenous HDAC species from extracts of human H1299 cells. In each case, we immunoprecipitated significant amounts of corresponding HDAC proteins. We determined the presence of pRB in these complexes by Western blot analysis using G3-245 monoclonal antibody that recognizes both hyperphosphorylated and hypophosphorylated forms of pRB. Fig. 5A shows that the hypophosphorylated form of pRB co-precipitated with HDAC1. Interestingly, we also detected significant amounts of hypophosphorylated pRB in association with HDAC2 and HDAC3. These interactions were specific as we did not detect pRB in immunoprecipitates prepared from the same cell extracts using antibody to CREB-binding protein (CBP), a histone acetyltransferase. These results indicate that the active form of pRB is able to recruit all three forms of HDACs.

HDAC1 also interacts with pRB-related family members p107 and p130 We immunoblotted immunoprecipitates from H1299 cells containing various HDACs with C-18 polyclonal antibody, which recognizes up to three different phosphorylated forms of p107 in various cell lines. Fig. 5B shows that p107, in particular its underphosphorylated forms, also co-precipitated with all endogenous HDAC species. We attempted a similar experiment with p130, but, as H1299 cells die upon serum starvation and p130 is expressed largely at growth arrest, we were unable to detect sufficient quantities. Significant amounts of p130 were present in asynchronized H1299 cells, but these p130 species are mostly hyperphosphorylated and did not appear to associate with HDACs at significant levels.

Adenovirus E1A protein and SV40 large T antigen associate with the

pRB pocket via LXCXE binding motifs and disrupt interactions between pRB and HDAC1. We therefore conducted a parallel series of binding studies in 293T cells, which express high levels of both Ad5 E1A proteins and SV40 large T antigen. Fig. 5A shows that no interactions were apparent between pRB and any of the HDAC enzymes, including HDAC3 in these cells. Fig. 5B shows that a similar effect was apparent with p107. Thus in both cases, binding of all three HDAC enzymes seemed to require the RB pocket region targeted by DNA tumor virus proteins.

Example 6: The small pocket of pRB interacts with different histone deacetylases

It has been proposed that HDAC1 utilizes a degenerate IXCXE motif to interact with the pocket (residues 379 to 792) of pRB (Ferreira et al., Proc. Natl. Acad. Sci. USA 95:10493-10498, 1998; Magnaghi-Jaulin et al., Nature 391:601-605, 1998). To determine directly if this region is involved in binding of all of the HDACs, we carried out studies using extracts from H1299 cells co-transfected with plasmid DNA expressing the small pocket of pRB as a fusion product with the DNA binding domain of Gal4 (Gal4DBD), termed Gal4-RB(pocket), as well as Flag-tagged versions of HDAC1, 2, or 3. Following immunoprecipitation using an antibody against Gal4DBD, we resolved precipitates by SDS-PAGE and, after transfer, analyzed the presence of HDAC1, 2, or 3 by Western blotting using anti-Flag antibody. Fig. 6A shows that all three HDACs, which were detected at similar levels in whole cell extracts, associated with the small pocket of pRB *in vivo*. Anti-Flag antibody recognized three Flag-HDAC1 species, but only the slowest migrating form was evident in Gal4-RB(pocket) precipitates, suggesting that the faster migrating species may be degradation products. We detected Flag-HDAC3 as two closely migrating species and both associated with the Gal4-RB(pocket).

These interactions were highly specific as a cDNA expressing Gal4DBD linked to VP16, a known transcriptional activator, did not associate with any of the histone deacetylases (Fig. 6A) even though Gal4-RB(pocket) and Gal4-VP16 were expressed at comparable levels. These results, as well as those described above with anti-CBP antibody, demonstrate clearly the specificity of interactions involving HDACs, and rule out the possibility that the interactions we observed were non-specific.

Fig. 6B shows that in a similar experiment in 293T cells, none of the HDAC proteins were present in immunoprecipitates containing Gal4-RB(pocket). Identical results were also obtained in 293 cells that express only Ad5 E1A proteins (Fig. 6C). Fig. 6D shows that addition prior to immunoprecipitation of increasing amounts of *in vitro* synthesized E1A protein fused to GST (GST-E1A) (Corbeil et al., J. Virol. 68:6697-6709, 1994) to extracts from H1299 cells expressing Gal4-pRB(pocket) and Flag-HDAC1 caused a decrease in binding of HDAC1 to the small pocket of pRB. We did not observe such an effect with GST/glutathione-Sepharose alone. Fig. 6D also demonstrates that pRB coimmunoprecipitated with E1A protein but not with GST. We obtained similar results using Flag-HDAC2 and Flag-HDAC3. Thus the small pocket of pRB is important for interactions not only with HDAC1, but also with HDAC2 and HDAC3. Disruption of such binding by E1A protein or T antigen also implied that the association of histone deacetylases may be mediated by LXCXE-like interactions. Although HDAC1 and HDAC2 contain IXCXE motifs, the mechanism of binding of HDAC3 was uncertain as HDAC3 lacks such sequences. Of further interest, no interaction was previously observed between the pocket of pRB and HDAC1 or HDAC2 in studies involving the yeast two-hybrid method (Brehm et al., Nature 391:597-601, 1998). As all three human histone deacetylases are highly homologous, it was possible that interactions of all three with the pocket may be indirect, and

involve an additional LXCXE-containing protein as a linker. One possible candidate is RBP1.

Example 7: RBP1 interacts with histone deacetylases

We tested the ability of RBP1 to associate with histone deacetylases. Fig. 7A shows that, when expressed in H1299 cells, Gal4-RBP1 (but not Gal4-VP16) interacted with all three histone deacetylases. Again, only the slowest migrating Flag-HDAC1 species was evident and Flag-HDAC3 was present as a doublet in Gal4-RBP1 precipitates. Fig. 7B shows that in 293T cells expressing E1A products and SV40 large T antigen, both HDAC1 and HDAC3 associated with Gal4RBP1, indicating that these interactions were unaffected by high levels of these viral pocket-binding proteins, and thus did not require the binding to RB. Although binding of HDAC2 to RBP1 was not evident in Fig. 7B, residual amounts of HDAC2 were detected after longer exposures of the blot. In addition, we observed a low level of binding of HDAC2 to RBP1 in a parallel experiment, shown in Fig. 6C, using 293 cells. Flag-HDAC2 expression was consistently low in 293T or 293 cells and this effect probably resulted from the fact that Flag-HDAC2 was expressed under a hybrid SV40/HTLV1 promoter, whereas Flag-HDAC1 and Flag-HDAC3 expression relied on the CMV promoter. Thus, we re-examined RBP1-HDAC binding in an additional experiment in which all three HDACs were expressed along with HA-tagged RBP1 in 293T cells as Gal4DBD-HDAC fusion products under the SV40 early promoter. Fig. 7C shows that all HDACs were expressed at comparable levels as determined by immunoblotting of whole cell extracts with anti-Gal4DBD antibody. Similarly, all cells expressed comparable amounts of RBP1, as detected using anti-HA antibody. Fig. 7C

with anti-HA antibody. We observed no such binding with Gal4 alone or with Gal4-VP16. Fig. 7D shows that interactions between RBP1 and endogenous HDAC enzymes could also be detected in H1299 cells. With immunoprecipitates prepared using the same polyclonal antibodies against individual HDACs employed in Figs. 5A and 5B, we found RBP1 in association with all three HDACs. RBP1 interactions with HDAC1 clearly occurred at much higher levels than with HDAC2 and HDAC3, implying that RBP1/HDAC1 complexes represent the predominant species in H1299 cells. This observation may suggest that RBP1 could be a component of the HDAC1 core complex. If this is the case, much of the endogenous RBP1/HDAC1 complex could be targeted to sites other than RB family members as the levels of RBP1 detected in association with these proteins were much lower than those observed with HDAC1. Fig. 7D also shows that RBP1 associates with all members of the RB family, and examination of pertinent lanes in Figs. 5A and 5B indicated that this association occurred preferentially with hypophosphorylated forms of pRB (Fig. 1A) and p107 (Fig. 1B). In addition, Fig. 7D shows that such RBP1-pRB/p107/p130 interactions were disrupted in 293T cells, as expected. Taken together, these results indicate that interactions between RBP1 and RB family members are truly pocket-dependent, as they were disrupted by T antigen or E1A pocket-binding proteins. To the contrary, interactions between RBP1 and HDACs were not sensitive to disruption by these viral pocket-binding proteins, suggesting that HDACs cannot be recruited to RBP1 via endogenous RB family members present in 293T cells. Instead, RBP1 could mediate the association between RB family members and all three HDAC enzymes.

It is unlikely that interactions of histone deacetylases with RBP1 occur indirectly through the recruitment of RB family members by RBP1. In 293T/293 cells, we failed to observe interactions either between the pocket of

pRB and these enzymes (Figs. 2B and 2C) or between RBP1 and RB family members (Figs. 1A, 1B, and 3D). In addition, we studied interactions between histone deacetylases and RBP1(ΔLCE). Fig. 7E shows that in 293T cells, both Gal4RBP1(ΔLCE) or RBP1(ΔLCE)-HA mutants interacted with Flag-tagged or Gal4DBD-tagged histone deacetylases. We obtained similar results in human H1299 cells. Thus, histone deacetylases appear to interact with RBP1 in a region apart from the pocket-binding motif, further supporting a role for RBP1 in bridging interactions between histone deacetylases and pRB/E2F complexes.

Example 8: RBP1 contains two independent transcriptional repression domains

As described herein, we identified a repression domain within RBP1. When Gal4 was fused to RBP1 having a deletion of this repression domain, referred to henceforth as R1, this mutant RBP1 product (Gal4-RBP1ΔR1) still repressed CAT expression at high levels (Fig. 8A), suggesting that a second repression domain may exist towards the carboxyl terminus of RBP1. We therefore generated a series of in-frame carboxyl terminal deletion mutants that also lacked R1 (illustrated in Fig. 8B) and found that all, including Gal4-ΔR1-93C that lacked only 93 residues at the carboxyl terminus, failed to repress CAT expression (Fig. 8A). We showed that all mutants used in experiments in Fig. 8 were expressed at similar high levels. As these data indicated that the second repression domain likely lies at the very carboxyl terminus, we generated three additional constructs that contained only varying amounts of the carboxyl terminus of RBP1 linked to the Gal4DBD. Fig. 8A shows that all three, Gal4-R2(1311-C), Gal4-R2(1314-C) and Gal4-R2(1263-C), repressed CAT expression. Thus, a second RBP1 repression domain (termed R2) exists between residues 1167-1257. Furthermore, repression by neither R1 nor R2 relies on the LXCXE pocket-binding motif

domain (termed R2) exists between residues 1167-1257. Furthermore, repression by neither R1 nor R2 relies on the LXCXE pocket-binding motif when RBP1 is tethered to DNA by a heterologous DNA binding domain like Gal4DBD.

Example 9: Transcriptional repression by RBP1 is both dependent and independent of histone deacetylases activity

The ability of the pocket to actively repress transcription relies on both histone deacetylase-dependent and -independent mechanisms. Only a subset of promoters repressed by the pocket of pRB were sensitive to the specific histone deacetylase inhibitor, trichostatin A (TSA). Among these, the only Gal4-dependent promoter/reporter construct found to be repressed by the pocket and to be sensitive to TSA is G5MLPCAT (containing the adenovirus major late promoter). Fig. 8C shows that both Gal4-RBP1 and Gal4-pRB(pocket) repressed the G5MLPCAT reporter, as did both Gal4-R1 and Gal4-R2 that contain only R1 and R2, respectively. Fig. 8C also shows that following treatment of transfected cells with 330nM TSA for 24 hours prior to harvesting, repression by both Gal4-pRB(pocket) and Gal4-RBP1 was partially relieved; however, whereas drug treatment completely abolished repression by Gal4-R2, however, it had no effect on that by Gal4-R1. Thus it appears that repression by R2 depends on histone deacetylases whereas that by R1 does not. We also noted that whereas Gal4-HDAC1 repression activity is completely relieved by TSA (Fig. 8C), repression by Gal4-Ad5-E1B-55K, an adenoviral repressor known to block p53-dependent transactivation (Yew et al., Genes Dev. 8:190-202, 1994; Teodoro et al., J. Virol. 71:3620-3627, 1997), at this promoter is not affected by TSA, suggesting that the adenovirus major late promoter can also be subject to both histone deacetylase-dependent and -independent repression. These results therefore indicate that RBP1 plays an important role in repression by pRB as TSA only partially relieves repression by the pRB pocket. Interestingly, TSA had little effect on repression by

Gal4-RBP1 or Gal4-R2 using either the G5TKCAT or G5SV40CAT promoters. We extended studies from the synthetic G5MLPCAT construct to the E2F-dependent promoter regulating E2F-1 expression (E2F1-luc). Fig. 8D shows that both RBP1 and the pocket of pRB repressed expression of luciferase from the E2F1-luc reporter, whereas RBP1 lacking the LXCXE pocket-binding motif or a pRB point mutant (C706F) did not. Addition of TSA partially relieved repression by both RBP1 and the pRB pocket. These results indicate that both the RBP1 histone deacetylase-dependent and -independent repression activities can repress this E2F-dependent promoter via interactions with RB family members, and thus interactions with RBP1 could provide both types of repression activities attributed to the pocket of RB family members (Luo et al., Cell 92:463-473, 1998).

Example 10: Only one repression domain of RBP1 interacts with histone deacetylases

We tested the ability of individual R1 and R2 transcriptional repression domains to interact with histone deacetylases. Fig. 9A shows results of co-immunoprecipitation experiments using 293T cells expressing Gal4-RBP1 constructs and Flag-HDAC3 in which we immunoprecipitated RBP1 with anti-Gal4DBD antibodies, then detected HDAC3 by immunoblotting with anti-Flag antibody. We did not observe any HDAC3 binding using either R1 or just the ARID portion of R1, but such binding was clearly evident with both R2 constructs and with RBP1 lacking R1. Binding was eliminated or greatly reduced using RBP1 lacking R2. We obtained similar results using Flag-HDAC1 and -HDAC2.

At present it is not known if histone deacetylases bind directly to the RBP1 or if they interact indirectly via an additional R2-binding protein. It is highly likely that RBP1 directly binds to another member of the protein

complex that contains an HDAC. The most likely candidate is the SAP30 adaptor protein, but other proteins (e.g., SinA and SinB) that are part of protein complexes that contain HDACs may also bind directly to RBP1 (Pazin and Kadonaga, Cell 89:325-328, 1997, hereby incorporated by reference). The actual direct binding partners can be determined, for example, by ascertaining whether *in vitro* translated protein pairs are capable of direct binding. We have discovered the presence of several proteins that co-precipitate with RBP1 using anti-RBP1 antibodies. One of the most prominent species was a protein of about 48kDa. HDAC1 copurifies with RbAp48 that, along with RbAp46, plays a role in targeting HDAC1 to histones (Zhang et al., Mol Cell 1:1021-31, 1998). It is possible that both RBP1 and RbAp48/46 could co-exist in a single HDAC complex. RbAp48 binds to the so-called "extended pocket" including a carboxy terminal portion of pRB and thus might play a role as a linker for HDAC1. RbAp48 lacks the LXCXE- binding motif, however, and thus is not targeted to the small pocket. In addition, no interactions have been detected using the yeast two-hybrid system between the pRB pocket and HDAC1 or HDAC2, even though yeast cells contain high levels of MSI1, a protein that is highly homologous to RbAp48/46 (Zhang et al., Mol Cell 1:1021-31, 1998). It is very likely, then, that RBP1 is responsible for bridging the pocket of RB family members to histone deacetylase complexes to repress a diversity of E2F-dependent promoters. RBP1 therefore appears to represent a major component of the growth regulatory machinery controlled by RB family members.

The above-described results were obtained using the following materials and methods.

Cell culture and transfection

Monkey CV-1 cells (ATCC CCL 70) and human lung carcinoma-

H1299 cells were grown in Dulbecco's modified eagle medium (D-MEM) containing 10% fetal calf serum. Chinese hamster ovary (CHO) cells (ATCC CCL-61) were grown in α-minimal essential medium supplemented with 10% fetal calf serum. Human cervical carcinoma (C33A; ATCC HTB-31), 293 cells, 293T cells (a variant of 293 cells that express SV40 large T antigen) and Chinese hamster ovary (CHO) cells (ATCC CCL-61) were grown in α-minimal essential medium supplemented with 10% fetal calf serum. In many experiments, CV-1 cells were induced to stop proliferating by incubating in D-MEM containing 2% dialyzed calf serum for 72 hrs, as described previously (Corbeil et al., Oncogene 15:657-668, 1997). Transfections were carried out by the calcium phosphate precipitation method (Graham and van der Eb, Virology 52:456-467, 1973) using pGEM plasmid as carrier DNA.

Plasmids

E2F1-luc(wt) and E2F-luc (E2F-) reporter constructs were described previously (Smith et al., Mol. Cell. Biol. 16:6965-6976, 1996). Mammalian plasmid expression vectors included pCMVRBP1-HA (provided by Bill Kaelin) and pCMVRBP1ΔLCE-HA (constructed by deleting residues 921-1056 of RBP1 coding region from pCMVRBP1-HA using restriction enzyme digests involving *Nsi*I and *Kpn*I). Plasmids expressing human pRB and p130, or the product of 12S Ad5 E1A mRNA were subcloned into pcDNA3 (Invitrogen). The G5TKCAT reporter construct has been described elsewhere (Yew et al., Genes Dev. 8:190-202, 1994; Teodoro et al., J. Virol. 71:3620-3627, 1997). Gal4-RBP1(SV40) plasmid was generated from pCMVRBP1-HA by polymerase chain reaction using Vent DNA polymerase (New England Biolabs, Mississaugua, ON) with primers specific for the RBP1 coding region. The amplified products were digested with *Sma*I and *Xba*I restriction enzymes and subcloned in-frame with the Gal4 DNA binding domain of pSG424, which

expresses Gal4 fusion proteins in mammalian cells under the SV40 promoter. Gal4-RBP1(CMV) plasmid was constructed by subcloning the *Hind*III digested fragment of Gal4-RBP1(SV40) plasmid into pcDNA3, which expresses proteins in mammalian cells under the CMV promoter. All the carboxyl-terminal deletion mutants of Gal4-RBP1 were generated from restriction enzyme digests of the Gal4-RBP1(SV40) plasmid and subcloned into a modified pcDNA3 construct containing stop codons inserted 3' of the multicloning cassette. Gal4-RBP1-ΔLCE plasmid was constructed by deleting residues 921-1056 of RBP1 coding region from Gal4-RBP1(SV40) using restriction enzymes *Nsi*I and *Kpn*I. Gal4-RBP1-Rep plasmid was constructed by subcloning the restriction enzyme digested fragment that correspond to residues 388-599 of the Gal4-RBP1 coding region into pSG424. Gal4-Ad5E1B55K plasmid was described previously (Teodoro and Branton, J. Virol. 71:3620-3627, 1997). Mammalian expression plasmid expressing Gal4-pRB(pocket) was provided by Tony Kouzarides (Brehm et al., Nature 391:597-601, 1998). Flag-HDAC1, 2, and 3 and Gal4-HDAC1, HDAC2, HDAC3 constructs have been described elsewhere (Yang et al., Proc. Natl. Acad. Sci. USA 93:12845-12850, 1996; Yang et al., J. Biol. Chem. 272:28001-28007, 1997). Gal4-VP16 was provided by Arnie Berk (Yew et al., Nature 357:82-85, 1992). The G5MLPCAT reporter was provided by Doug Dean (Luo et al., Cell 92:463-473, 1998). Mutants of Gal4DBD-RBP1 were generated as follows: Gal4-ΔR1 mutants were generated using two specific primers close to the 3' end of the R1 region and the end of RBP1 coding sequence. PCR was done to generate fragments with unique restriction sites (*Bsp1107*I, *Hind*III) which were subcloned into digested RBP1 constructs in which all RBP1 coding sequences between the beginning of R1 to the end of the protein had been removed using the same restriction enzymes. All carboxy terminal deletion mutants of Gal4-ΔR1 were generated from fragments

produced by restriction enzyme digestion of the Gal4-ΔR1 plasmid DNA and these were then subcloned into a modified pcDNA3 (Invitrogen) construct containing stop codons inserted 3' of the multicloning cassette. Gal4-R2 truncation mutants were constructed by subcloning the restriction enzyme-digested fragments that correspond to residues 1311-1404, or 1314-1404, or 1263-1404 of the Gal4-RBP1 coding region into pSG424. Mammalian expression plasmids encoding the pocket of pRB and the inactive pRB pocket (C706F) mutant have been described previously (Kaelin et al., Cell 70:351-364, 1992). All constructs made were confirmed by sequencing using specific primers.

Cell Lysis and electrophoretic mobility shift assay(EMSA)

CV-1 cells were harvested and whole cell extracts were prepared using lysis buffer (50 mM HEPES-KOH, pH 7.9, containing 0.4 M KCl, 0.1%(v/v) NP-40, 4 mM NaF, 4 mM $NaVO_4$, 0.2 mM EDTA, 0.2 mM EGTA, 10% Glycerol (v/v), 1 mM DTT, 0.5 mM PMSF, 1 μg/mL pepstatin, 1 μg/mL leupeptin and 1 μg/mL aprotinin) and centrifuged for 45 min at 20,000 x g (Corbeil et al., Oncogene 11:909-920, 1995; Corbeil et al., Oncogene 15:657-668, 1997). EMSA was performed using whole cell extracts and an E2F-specific oligonucleotide (GATTTAAGTTTCGCGCCCTTTCTCAA (SEQ ID NO: 1)) and double stranded oligonucleotides were labeled using [α^{32}P]dCTP and Klenow polymerase. EMSA was carried out in 20 μL reaction mixtures containing 10-20 μg of whole cell extract in 20 mM HEPES-KOH (pH 7.9) containing 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.4 mM DTT, 4 μg BSA, 2.5% (v/v) Ficoll, 1-2 μg of salmon sperm DNA, and labeled probe containing 20,000-50,000 cpm. Antibody supershift experiments were performed by incubating extracts for 10 min on ice with 1-2 μg of antisera recognizing p130, pRB, p107, or RBP1. Reactions were incubated at room

temperature for 30 min and then samples were loaded on a 4% polyacrylamide gel (30:1 polyacrylamide: bis-acrylamide) in 0.25X TBE and subjected to electrophoresis at 200 V for 2 hr. Gels were dried and analyzed by autoradiography using Kodak X-omat film.

Antibodies

A rabbit polyclonal antibody raised against HDAC2 was described previously (Laherty et al., Cell 89:349-356, 1997). Antibodies against HDAC1 and HDAC3 were prepared against peptides corresponding to the unique carboxy termini of HDAC1 (EEKPEAKGVKEEVKLA) (SEQ ID NO: 2) and HDAC3 (NEFYDGDHDNDKESDVEI) (SEQ ID NO: 3) coupled to KLH and injected separately into New Zealand white rabbits. The resulting antibodies were immunoaffinity purified on peptide columns. Anti-pRB antibody G3-245 was purchased from PharMingen (San Diego, CA). Antibodies against p130 (C-20), p107 (C-18), and Gal4DBD (RK5C1) were purchased from Santa Cruz, anti-HA antibody HA.11 was purchased from BAbCo (Richmond, CA) and anti-Flag antibody M2 from Sigma, (St. Louis, MO).

Chloramphenicol acetyltransferase (CAT), ß-galactosidase and luciferase assays

CAT and ß-galactosidase assays were performed as described previously (Teodoro and Branton, J. Virol. 71:3620-3627, 1997). Luciferase assays were performed as described elsewhere (Wrana et al., Cell 71:1003-1014, 1992).

Colony formation assay

CV-1 cells were transfected with 3 µg of DNA from plasmids expressing neo as well as RBP1, including pRcCMVRBP1-HA (encoding an HA-tagged version of RBP1), pcDNA3Gal4-RBP1, and pcDNA3Gal4-RBP1ΔLCE. Cells were plated in medium containing 500 µg/mL of G418,

allowed to grow for two weeks, and then stained with crystal violet.

Binding assays

1 μg of cDNA encoding Gal4DBD, fused with the pocket of pRB, RBP1, or mutated RBP1, were introduced using lipofectamine (NEN Life Science, Boston, MA) along with 1 μg of those encoding Flag-tagged HDAC1, HDAC2, or HDAC3 into H1299 or 293T cells. In some experiments, 1 μg of cDNAs encoding HA- tagged RBP1 or RBP1 mutants were introduced using lipofectamine along with 1 μg of those encoding Gal4DBD fused HDAC1, HDAC2, or HDAC3 into H1299 or 293T. Cells were harvested 40 hours post-transfection and lysed with "low stringency buffer." Cell extracts were diluted to 150mM KCl in a 1 mL volume and precleared with protein G-Sepharose (Pharmacia, Baie D'Urfe, QC) for 2 hours. Precleared extracts were incubated with 1 μg of RK5C1-Gal4DBD antibody (Santa Cruz) and 30 μL of a 50% slurry of protein G-Sepharose for at least 12 hours. Immunoprecipitated Gal4-tagged protein complexes were washed six times with lysis buffer and eluted by boiling in 2X sample buffer. Eluted proteins were subjected to SDS-PAGE using a 10% polyacrylamide gel, and proteins were then transferred to PVDF membranes (Millipore, Bedford, MA) which were probed with either anti-HA or anti-Flag antibody and then by HRP-conjugated goat anti-mouse (light chain specific) secondary antibody. Binding was detected by Enhanced Luminol Reagent (NEN Life Science).

Other Embodiments

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described invention will be apparent to those skilled in the art. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not

be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the invention.

What is claimed is:

1. A method for modulating proliferation of a cell, said method comprising contacting said cell with an RBP1 polypeptide or a compound that modulates RBP1 biological activity or expression.

2. The method of claim 1, wherein said cell proliferation is decreased.

3. A method for identifying a compound that modulates cell proliferation, said method comprising contacting a cell with a candidate compound and monitoring the level of expression of an RBP1 polypeptide in said cell, wherein a change in said level of expression in response to said candidate compound, relative to the level of expression in a cell not contacted with said candidate compound, identifies said compound as one that modulates cell proliferation.

4. A method for identifying a compound that modulates cell proliferation, said method comprising:

(a) providing an RBP1 polypeptide having biological activity;

(b) contacting said RBP1 polypeptide with a candidate compound; and

(c) monitoring the level of said biological activity of said RBP1 polypeptide, wherein a change in said level of biological activity in response to said candidate compound, relative to the level of biological activity of an RBP1 polypeptide not contacted with said candidate compound, identifies said compound as a compound that modulates cell proliferation.

5. The method of claim 4, wherein said RBP1 polypeptide is in a cell.

6. The method of claim 1 or 5, wherein said cell is in a mammal.

7. The method of claim 6, wherein said mammal is a human or a rodent.

8. The method of claim 4, wherein said RBP1 polypeptide is in a cell-free system.

9. A method for determining whether a patient has a cell proliferation disease or an increased likelihood of developing said disease, said method comprising measuring the level of RBP1 biological activity in a sample from the mammal, wherein an alteration in said level of said biological activity, relative to a level of RBP1 biological activity in a matched sample from an unaffected person, indicates that the patient has said disease or increased likelihood of developing said disease.

10. The method of claim 1, 4, or 9, wherein said biological activity is binding of said RBP1 polypeptide to an E2F protein or repression of E2F-mediated transcription.

11. The method of claim 10, wherein said E2F is E2F-1, E2F-2, E2F-3, E2F-4, or E2F-5.

12. The method of claim 1, 4, or 9, wherein said biological activity is binding of said RBP1 polypeptide to an HDAC or a protein that is part of a complex containing an HDAC.

13. The method of claim 12, wherein said protein SAP30, SinA, or SinB.

14. A method for determining whether a patient has a cell proliferation

disease or an increased likelihood of developing said disease, said method comprising measuring the level of expression of an RBP1 polypeptide in a sample from said mammal, wherein an alteration in said level of expression of said RBP1 polypeptide, relative to a matched sample from an unaffected person, indicates that the patient has a cell proliferation disease or increased likelihood of developing said disease.

15. A method of repressing transcription in a cell, said method comprising introducing into said cell a polypeptide comprising: (i) a repressor domain from RBP1, and (ii) a DNA binding domain.

16. A polypeptide comprising: (i) a repressor domain from RBP1, and (ii) a DNA binding domain.

17. The polypeptide of claim 16, wherein said repressor domain comprises amino acids 241-452 or 1167-1257 of SEQ ID NO: 4.

18. A nucleic acid molecule encoding a polypeptide comprising: (i) a repressor domain from RBP, and (ii) a DNA binding domain.

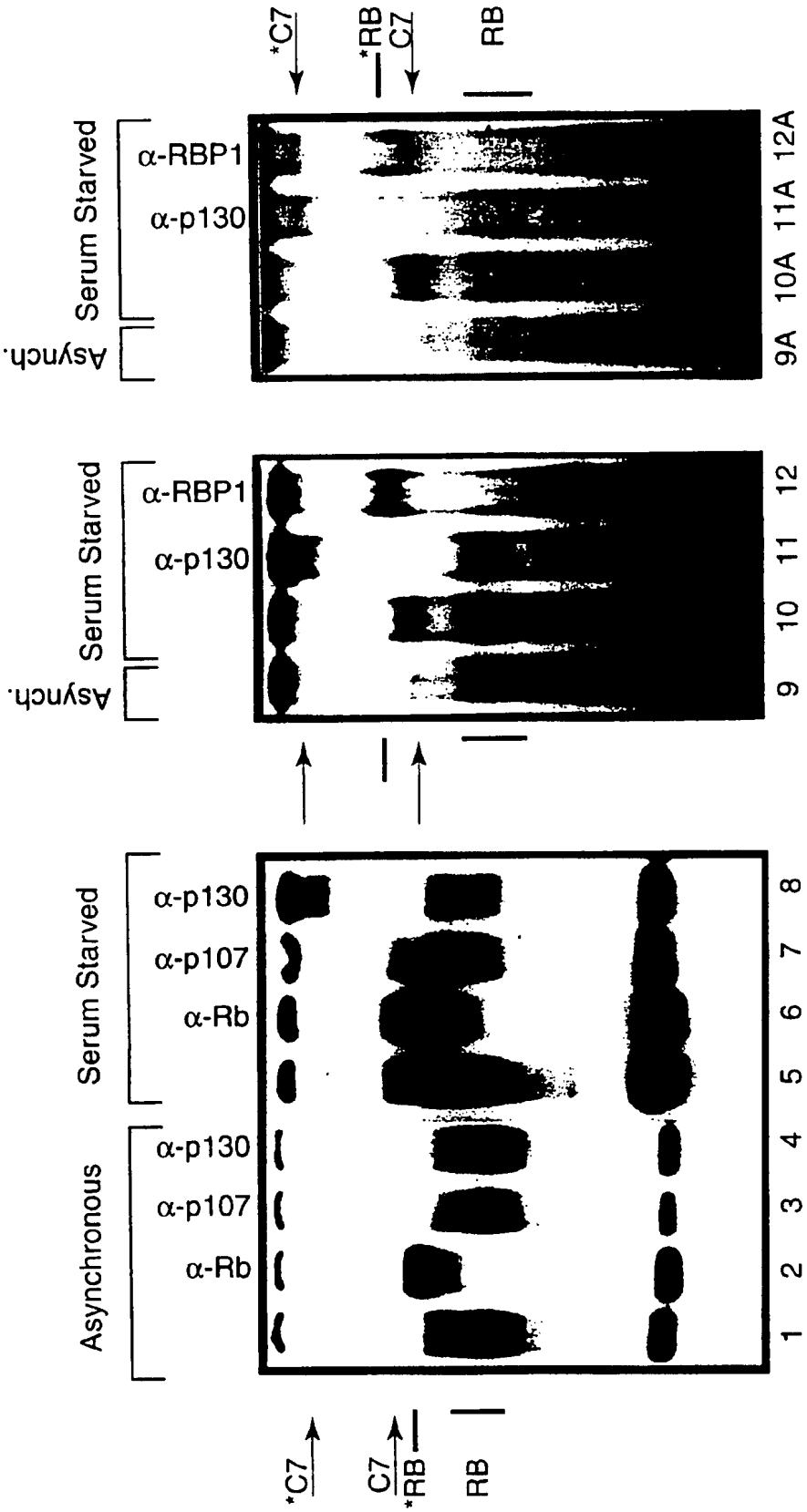
19. A composition comprising (i) a substantially purified RBP1 polypeptide, a substantially purified nucleic acid encoding an RBP1 polypeptide, or a substantially purified antibody that specifically binds an RBP1 polypeptide, and (ii) a physiologically acceptable carrier.

20. A kit for determining whether a human has a cell proliferation disease or an increased likelihood of developing said disease, said kit comprising a substantially pure antibody that specifically binds an RBP1

polypeptide.

21. The kit of claim 20, wherein said kit further comprises a means for detecting said binding of said antibody to said RBP1 polypeptide.

22. Use of an RBP1 polypeptide, a nucleic acid molecule encoding an RBP1 polypeptide, or an antibody that specifically binds to an RBP1 polypeptide for preparing a medicament for the treatment of a cell proliferation disease.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*RB
C7
RB
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α-RBP1
α-p130
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α-p130
Asynch.
9 10 11 12
Serum Starved
α-p130
α-p107
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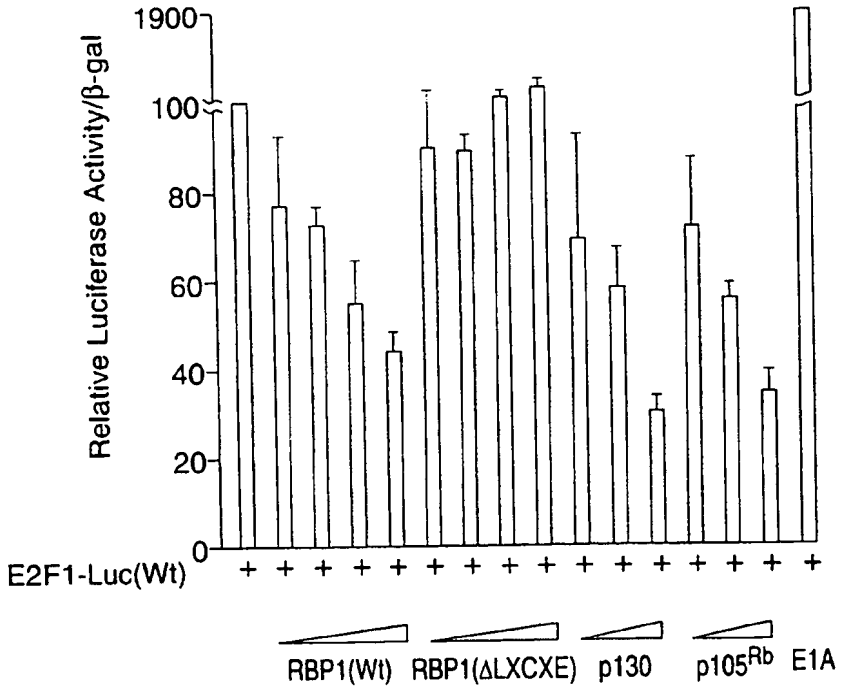
*C7
C7
*RB
RB

Fig. 1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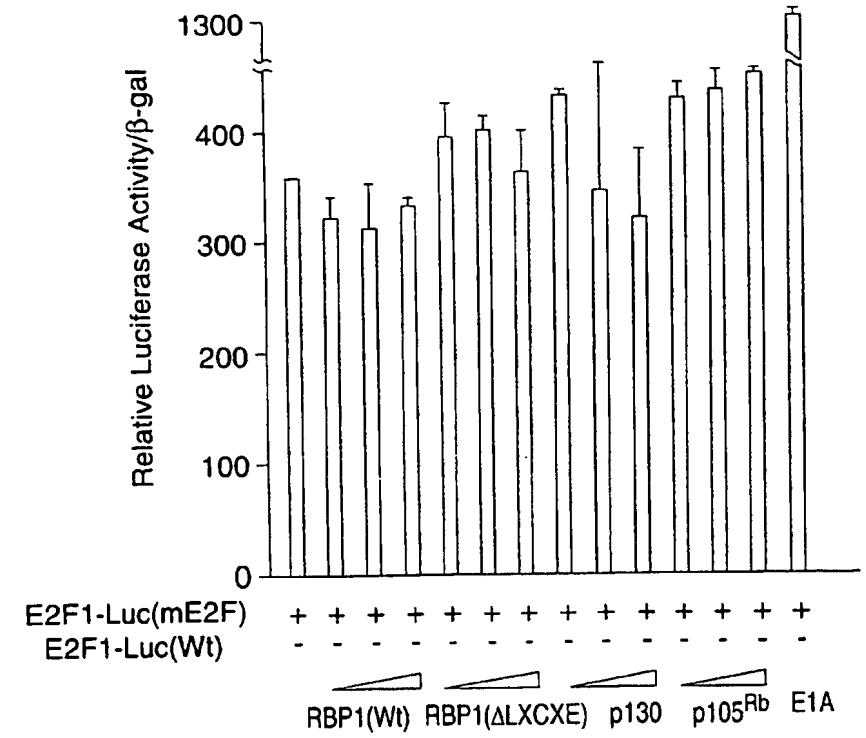
100
80
60
40
20
0
E2F1-Luc(Wt)
+ + + + + + + + + + + + + + + +
RBP1(Wt)
RBP1(ΔLXCXE)
p130
p105Rb
E1A

Fig. 2A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400
300
200
100
0
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+ + + + + + + + + + + + + + +
E2F1-Luc(Wt)
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RBP1(Wt)
RBP1(ΔLXCXE)
p130
p105Rb
E1A

Fig. 2B

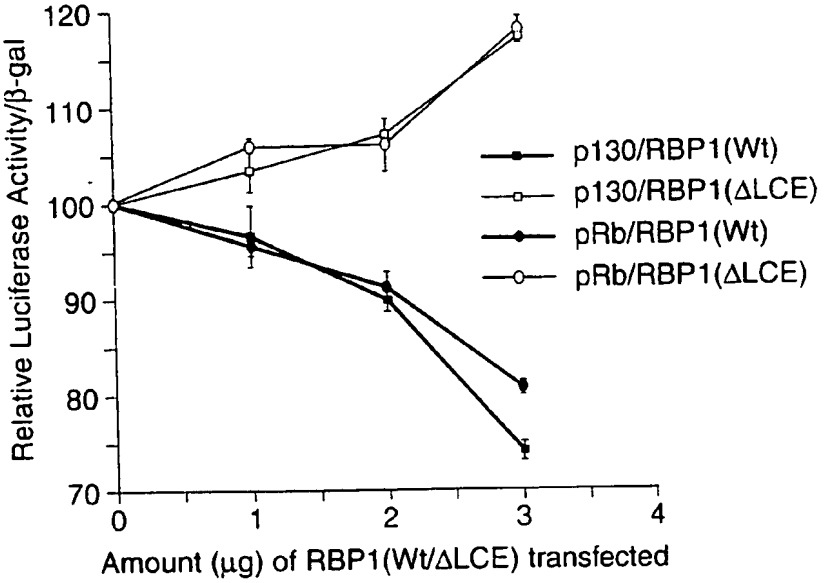

Relative Luciferase Activity/β-gal
120
110
100
90
80
70
0
1
2
3
4
Amount (μg) of RBP1(Wt/ΔLCE) transfected
p130/RBP1(Wt)
p130/RBP1(ΔLCE)
pRb/RBP1(Wt)
pRb/RBP1(ΔLCE)

Fig. 2C

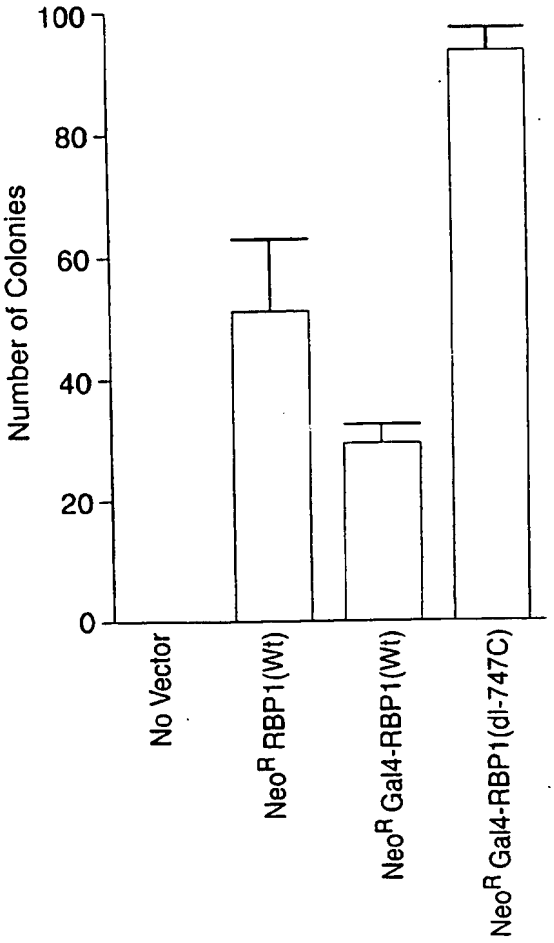

Number of Colonies
100
80
60
40
20
0
No Vector
NeoR RBP1(Wt)
NeoR Gal4-RBP1(Wt)
NeoR Gal4-RBP1(dl-747C)

Fig. 3A

pcDNA3

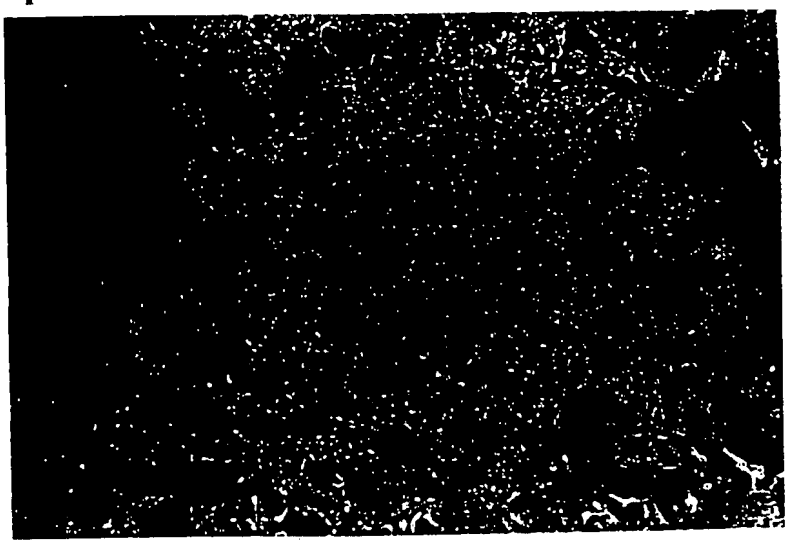


Fig. 3B

pRcCMV-RBP1-HA

Fig. 3C

pcDNA3Gal4-RBP1-dl-747 C

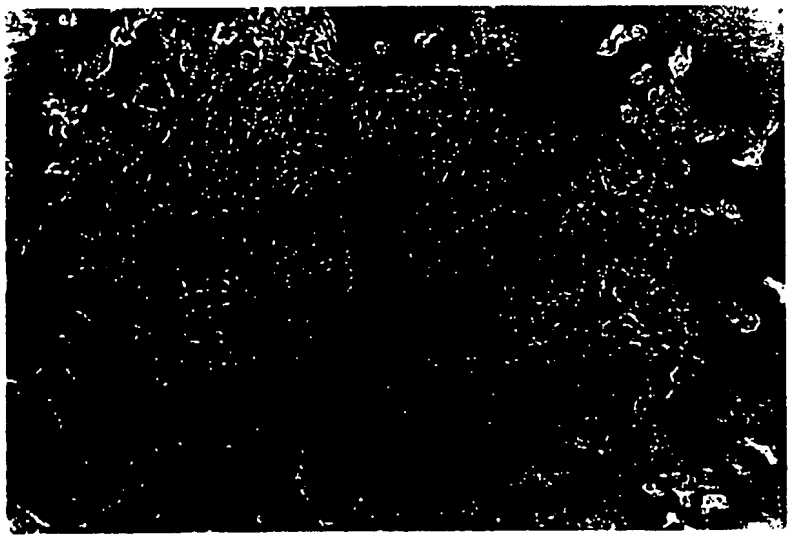


Fig. 3D

pcDNA3Gal4-RBP1

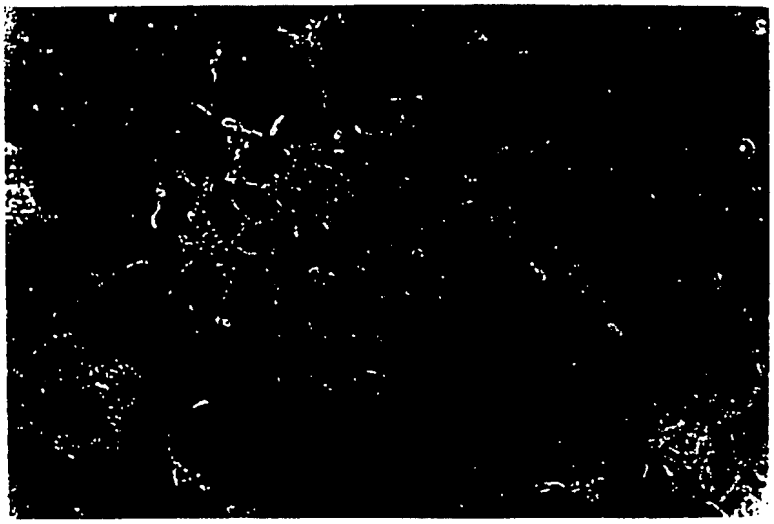


Fig. 3E

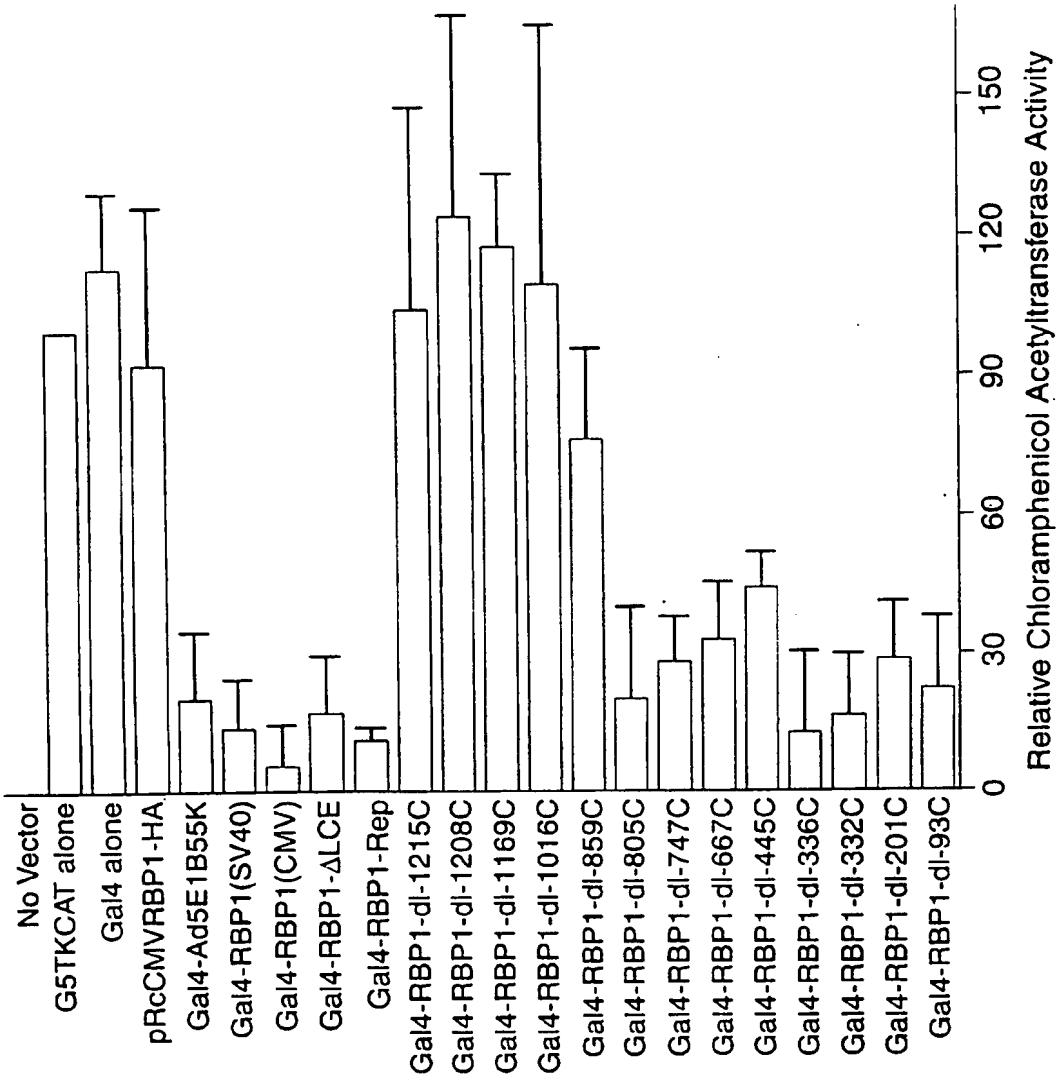
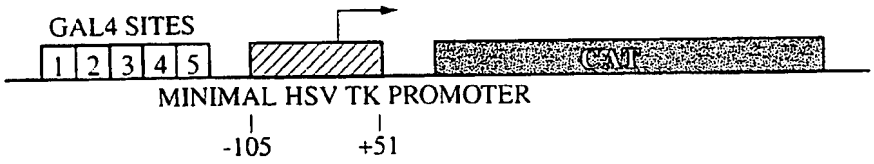

No Vector
G5TKCAT alone
Gal4 alone
pRcCMVRBP1-HA
Gal4-Ad5E1B55K
Gal4-RBP1(SV40)
Gal4-RBP1(CMV)
Gal4-RBP1-ΔLCE
Gal4-RBP1-Rep
Gal4-RBP1-dl-1215C
Gal4-RBP1-dl-1208C
Gal4-RBP1-dl-1169C
Gal4-RBP1-dl-1016C
Gal4-RBP1-dl-859C
Gal4-RBP1-dl-805C
Gal4-RBP1-dl-747C
Gal4-RBP1-dl-667C
Gal4-RBP1-dl-445C
Gal4-RBP1-dl-336C
Gal4-RBP1-dl-332C
Gal4-RBP1-dl-201C
Gal4-RBP1-dl-93C
0
30
60
90
120
150
Relative Chloramphenicol Acetyltransferase Activity

Fig. 4A


GAL4 SITES
1 2 3 4 5
CAT
MINIMAL HSV TK PROMOTER
-105
+51

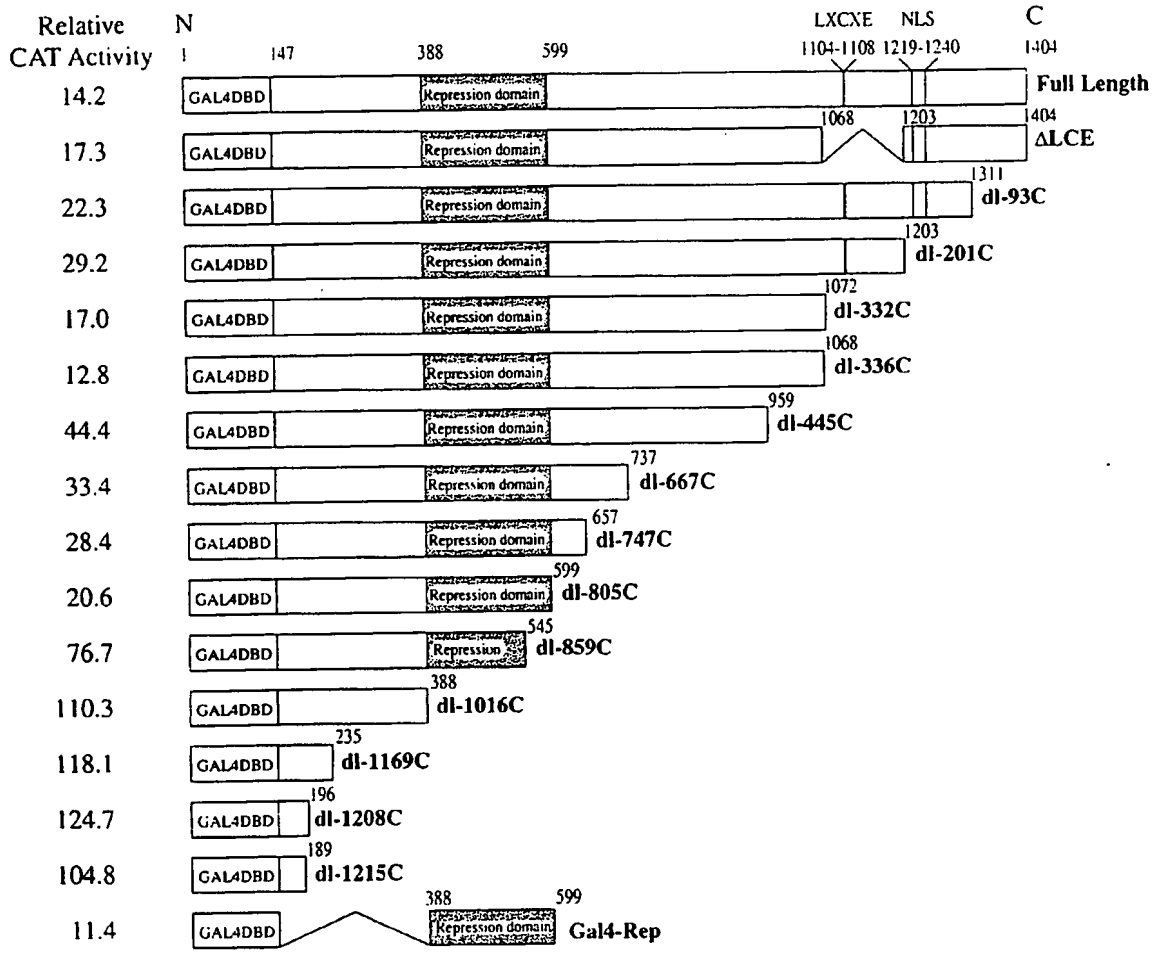

Relative CAT Activity
N
C
LXCXE
NLS
1 147 388 599 1104-1108 1219-1240 1404
14.2 GAL4DBD Repression domain Full Length
17.3 GAL4DBD Repression domain 1068 1203 1404 ΔLCE
22.3 GAL4DBD Repression domain 1311 dl-93C
29.2 GAL4DBD Repression domain 1203 dl-201C
17.0 GAL4DBD Repression domain 1072 dl-332C
12.8 GAL4DBD Repression domain 1068 dl-336C
44.4 GAL4DBD Repression domain 959 dl-445C
33.4 GAL4DBD Repression domain 737 dl-667C
28.4 GAL4DBD Repression domain 657 dl-747C
20.6 GAL4DBD Repression domain 599 dl-805C
76.7 GAL4DBD Repression 545 dl-859C
110.3 GAL4DBD 388 dl-1016C
118.1 GAL4DBD 235 dl-1169C
124.7 GAL4DBD 196 dl-1208C
104.8 GAL4DBD 189 dl-1215C
11.4 GAL4DBD 388 599 Repression domain Gal4-Rep

Fig. 4B

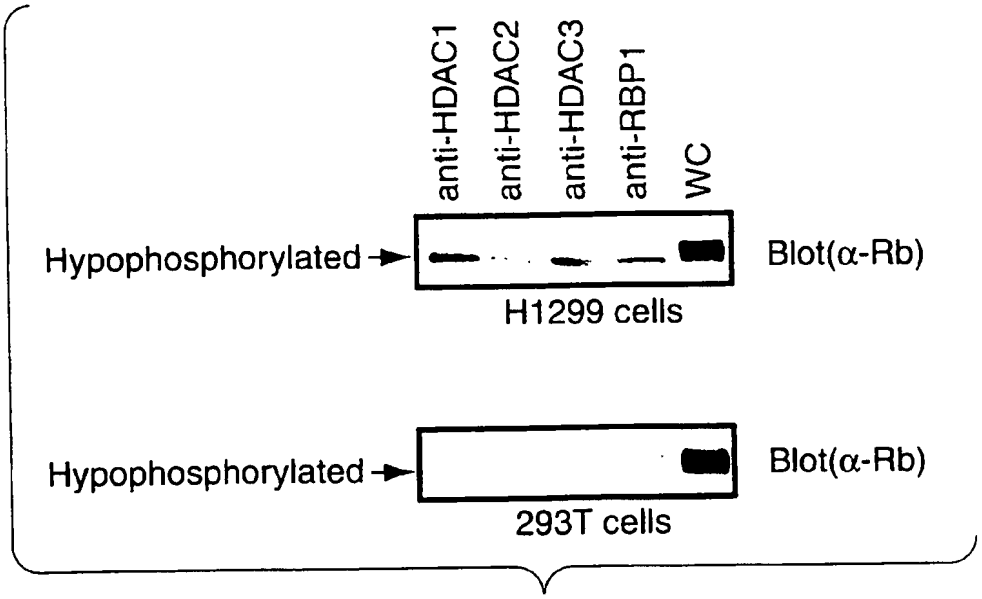

anti-HDAC1
anti-HDAC2
anti-HDAC3
anti-RBP1
WC
Hypophosphorylated
Blot(α-Rb)
H1299 cells
Hypophosphorylated
Blot(α-Rb)
293T cells

Fig. 5A

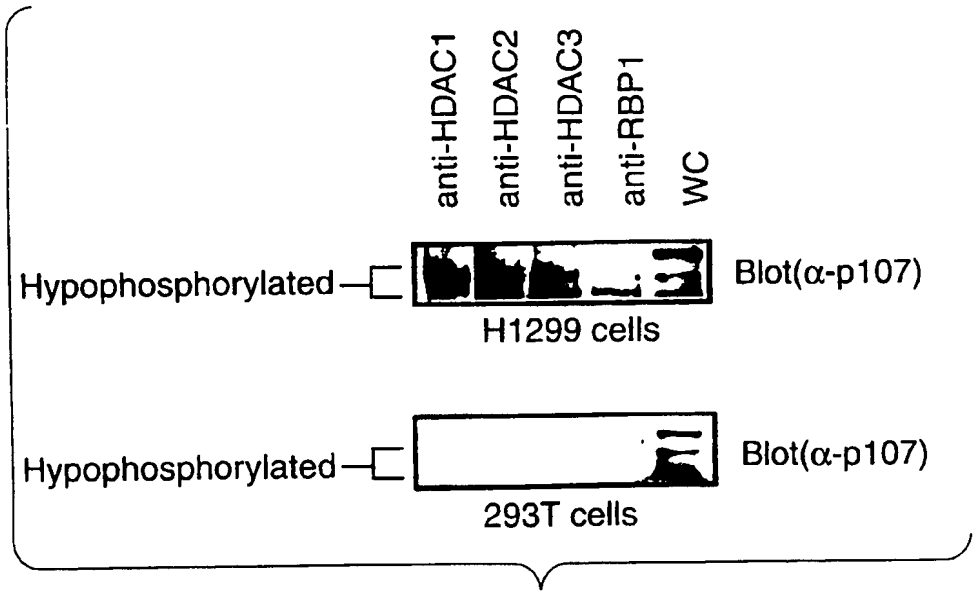

anti-HDAC1
anti-HDAC2
anti-HDAC3
anti-RBP1
WC
Hypophosphorylated
Blot(α-p107)
H1299 cells
Hypophosphorylated
Blot(α-p107)
293T cells

Fig. 5B

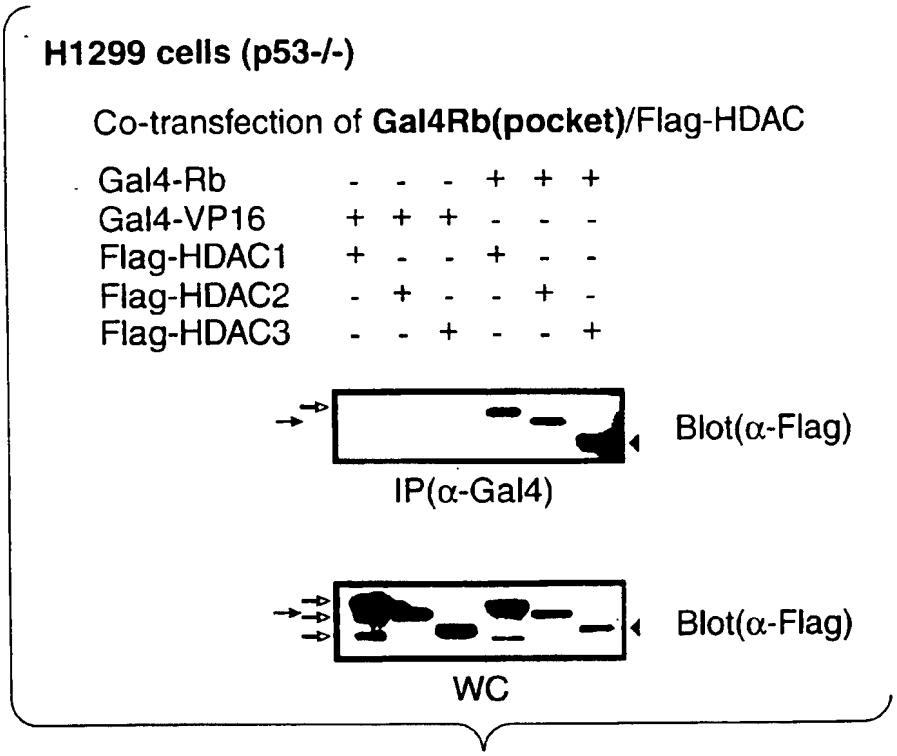

H1299 cells (p53-/-)
Co-transfection of Gal4Rb(pocket)/Flag-HDAC
Gal4-Rb - - - + + +
Gal4-VP16 + + + - - -
Flag-HDAC1 + - - + - -
Flag-HDAC2 - + - - + -
Flag-HDAC3 - - + - - +
Blot(α-Flag)
IP(α-Gal4)
Blot(α-Flag)
WC

Fig. 6A

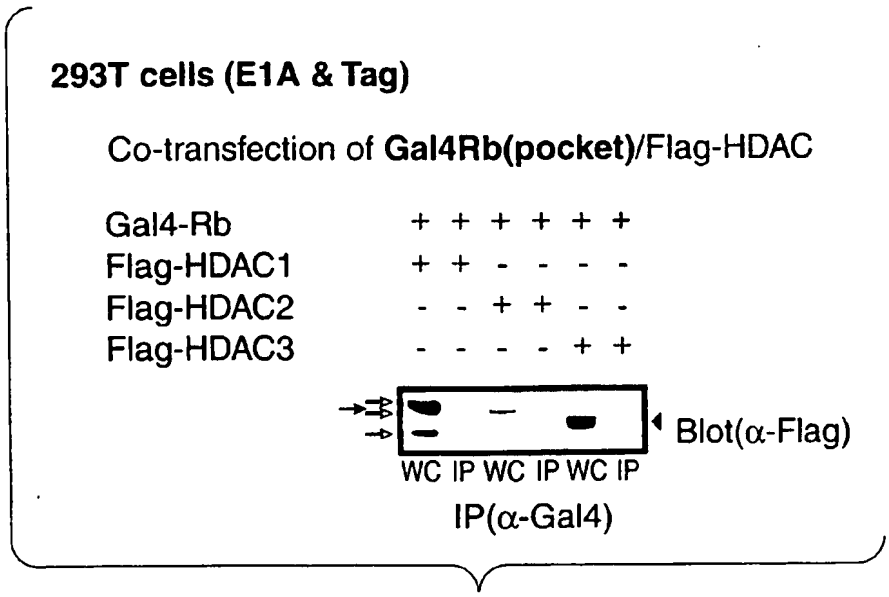

293T cells (E1A & Tag)
Co-transfection of Gal4Rb(pocket)/Flag-HDAC
Gal4-Rb + + + + + +
Flag-HDAC1 + + - - - -
Flag-HDAC2 - - + + - -
Flag-HDAC3 - - - - + +
Blot(α-Flag)
WC IP WC IP WC IP
IP(α-Gal4)

Fig. 6B

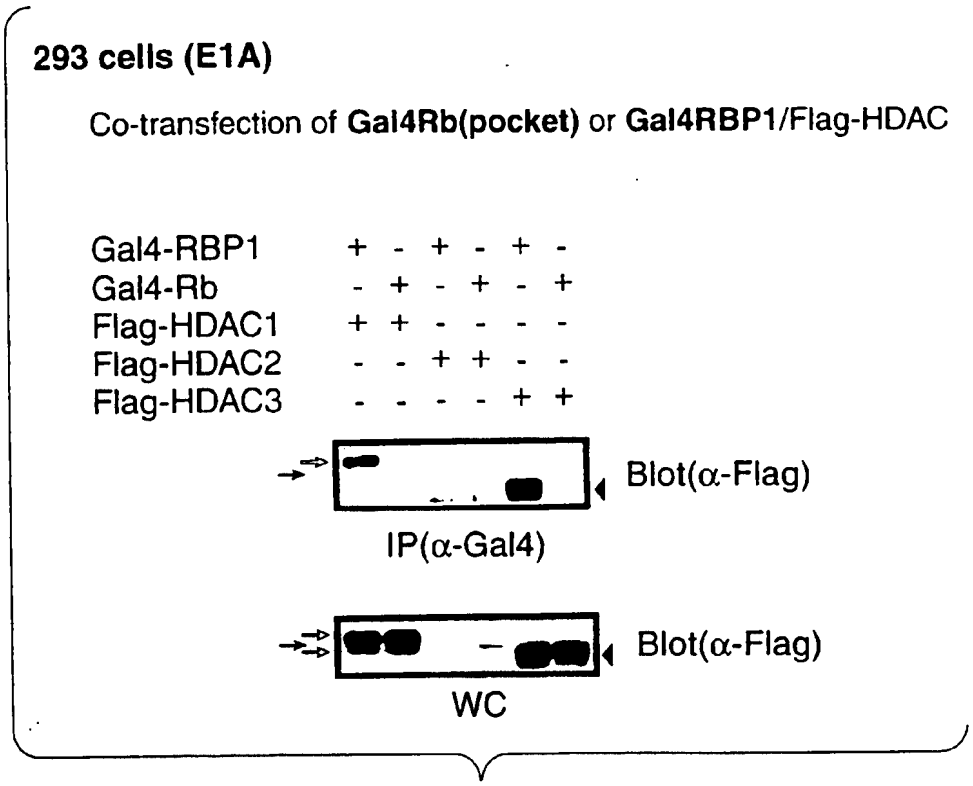

293 cells (E1A)
Co-transfection of Gal4Rb(pocket) or Gal4RBP1/Flag-HDAC
Gal4-RBP1 + - + - + -
Gal4-Rb - + - + - +
Flag-HDAC1 + + - - - -
Flag-HDAC2 - - + + - -
Flag-HDAC3 - - - - + +
Blot(α-Flag)
IP(α-Gal4)
Blot(α-Flag)
WC

Fig. 6C

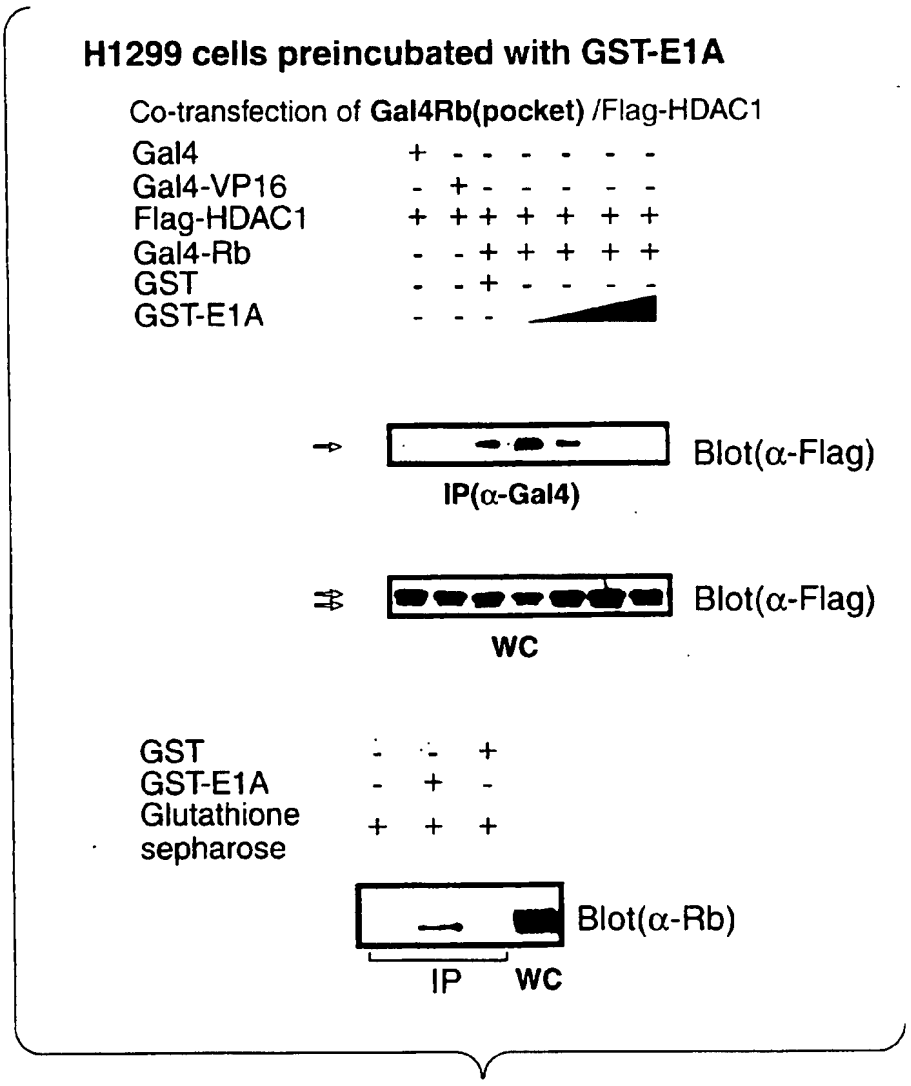

H1299 cells preincubated with GST-E1A
Co-transfection of Gal4Rb(pocket) /Flag-HDAC1
Gal4 + - - - - - -
Gal4-VP16 - + - - - - -
Flag-HDAC1 + + + + + + +
Gal4-Rb - - + + + + +
GST - - + - - - -
GST-E1A - - -
Blot(α-Flag)
IP(α-Gal4)
Blot(α-Flag)
WC
GST - - +
GST-E1A - + -
Glutathione sepharose + + +
Blot(α-Rb)
IP
WC

Fig. 6D

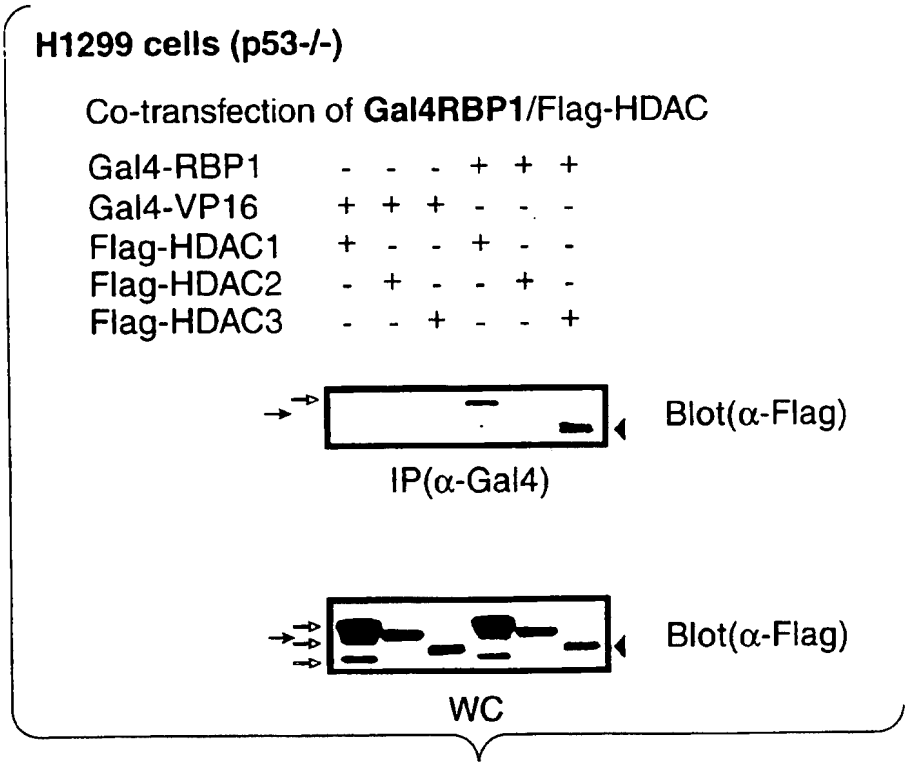

H1299 cells (p53-/-)
Co-transfection of Gal4RBP1/Flag-HDAC
Gal4-RBP1 - - - + + +
Gal4-VP16 + + + - - -
Flag-HDAC1 + - - + - -
Flag-HDAC2 - + - - + -
Flag-HDAC3 - - + - - +
Blot(α-Flag)
IP(α-Gal4)
Blot(α-Flag)
WC

Fig. 7A

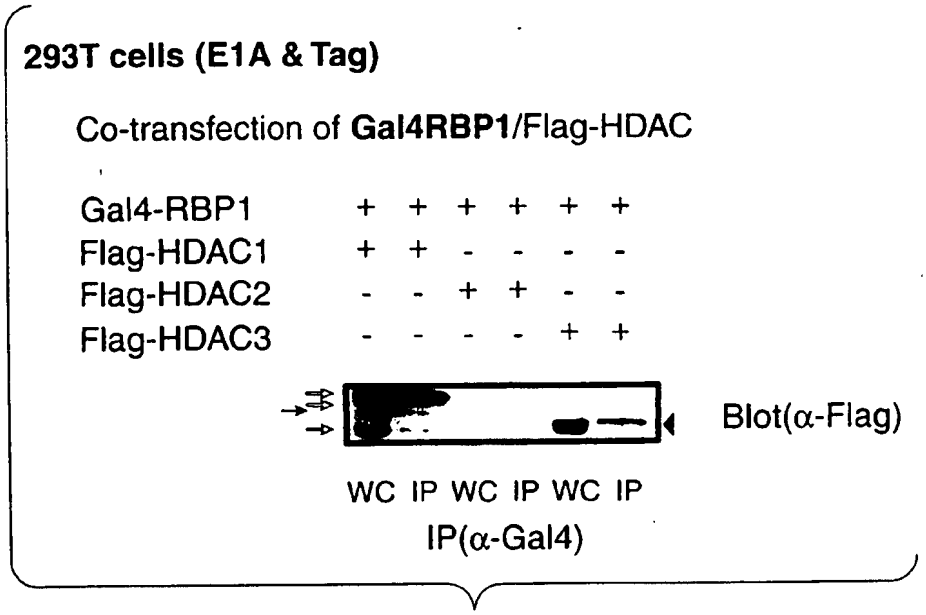

293T cells (E1A & Tag)
Co-transfection of Gal4RBP1/Flag-HDAC
Gal4-RBP1 + + + + + +
Flag-HDAC1 + + - - - -
Flag-HDAC2 - - + + - -
Flag-HDAC3 - - - - + +
Blot(α-Flag)
WC IP WC IP WC IP
IP(α-Gal4)

Fig. 7B

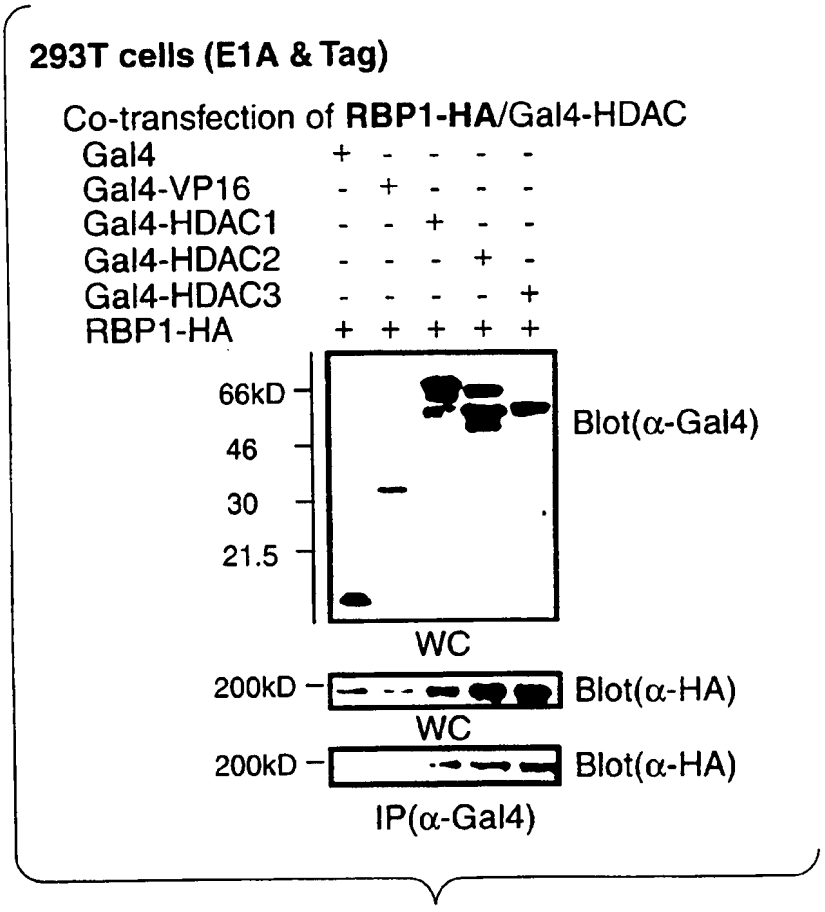

293T cells (E1A & Tag)
Co-transfection of RBP1-HA/Gal4-HDAC
Gal4 + - - - -
Gal4-VP16 - + - - -
Gal4-HDAC1 - - + - -
Gal4-HDAC2 - - - + -
Gal4-HDAC3 - - - - +
RBP1-HA + + + + +
66kD
46
30
21.5
Blot(α-Gal4)
WC
200kD
Blot(α-HA)
WC
200kD
Blot(α-HA)
IP(α-Gal4)

Fig. 7C

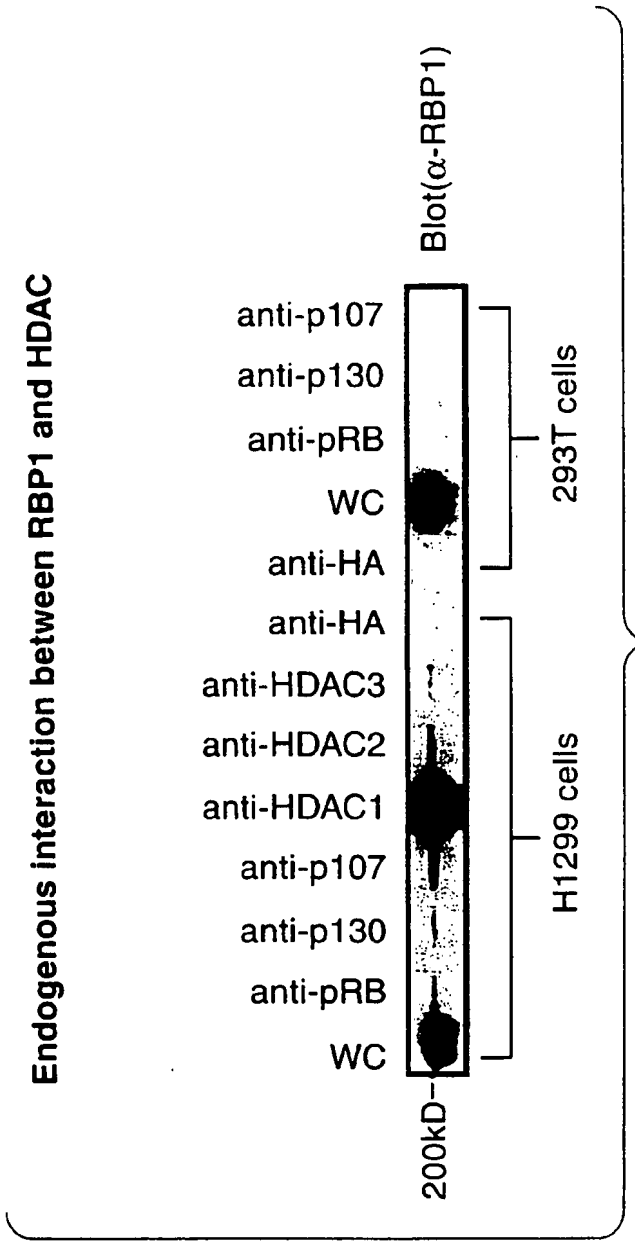

Endogenous interaction between RBP1 and HDAC
Blot(α-RBP1)
anti-p107
anti-p130
anti-pRB
WC
anti-HA
anti-HA
anti-HDAC3
anti-HDAC2
anti-HDAC1
anti-p107
anti-p130
anti-pRB
WC
200kD
293T cells
H1299 cells

Fig. 7D

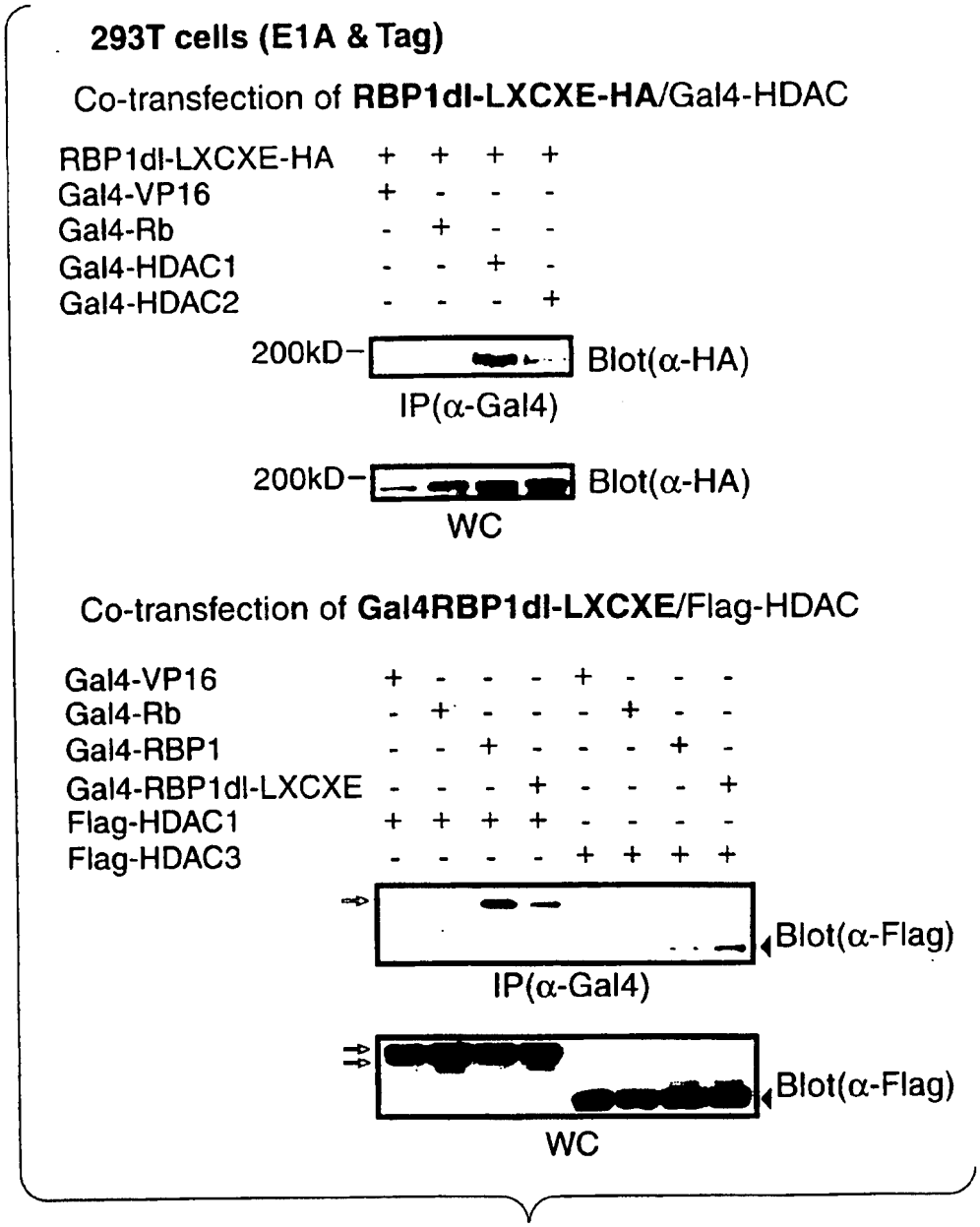

293T cells (E1A & Tag)
Co-transfection of RBP1dl-LXCXE-HA/Gal4-HDAC
RBP1dl-LXCXE-HA + + + +
Gal4-VP16 + - - -
Gal4-Rb - + - -
Gal4-HDAC1 - - + -
Gal4-HDAC2 - - - +
200kD
Blot(α-HA)
IP(α-Gal4)
200kD
Blot(α-HA)
WC
Co-transfection of Gal4RBP1dl-LXCXE/Flag-HDAC
Gal4-VP16 + - - - + - - -
Gal4-Rb - + - - - + - -
Gal4-RBP1 - - + - - - + -
Gal4-RBP1dl-LXCXE - - - + - - - +
Flag-HDAC1 + + + + - - - -
Flag-HDAC3 - - - - + + + +
Blot(α-Flag)
IP(α-Gal4)
Blot(α-Flag)
WC

Fig. 7E

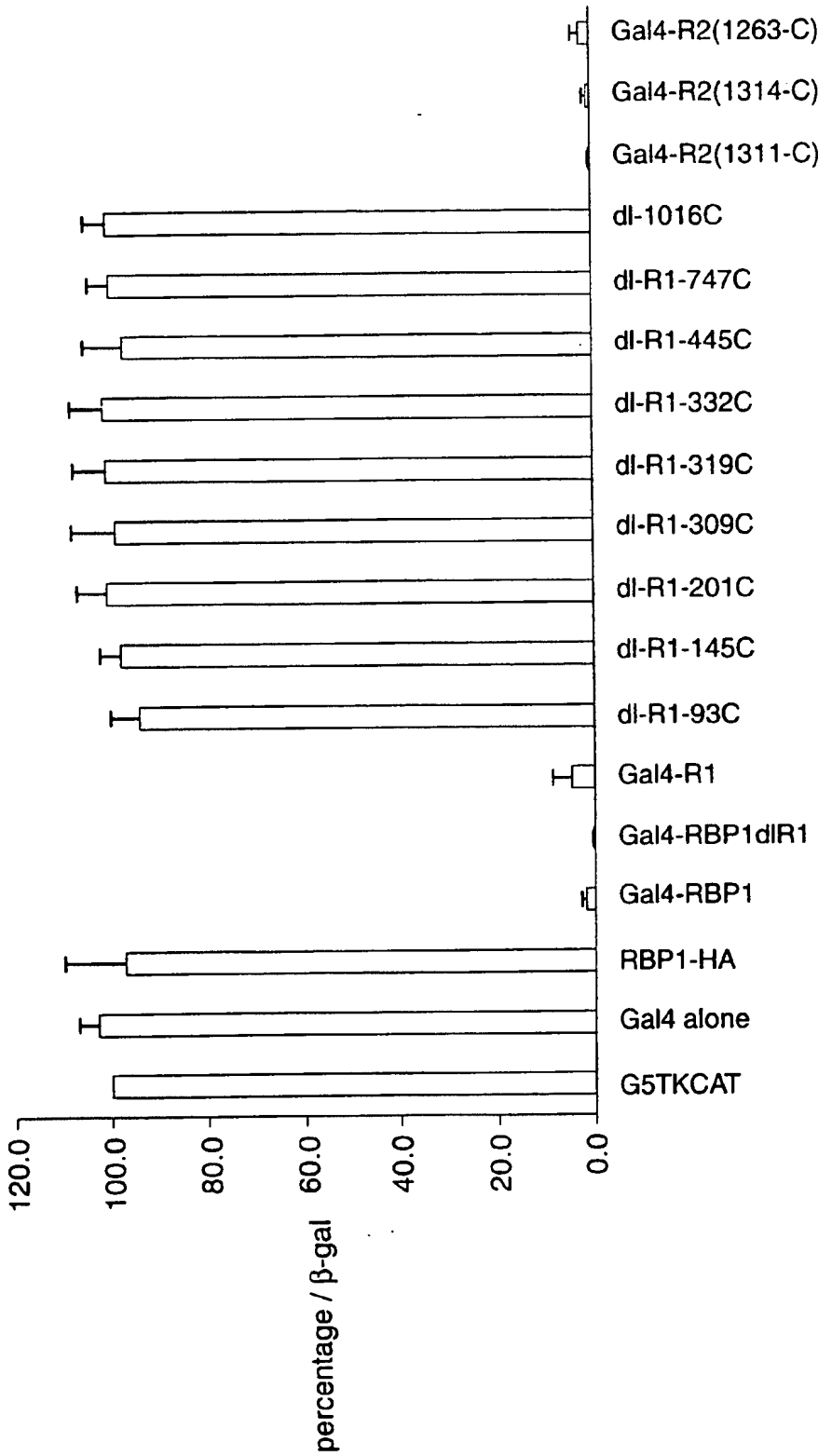

Gal4-R2(1263-C)
Gal4-R2(1314-C)
Gal4-R2(1311-C)
dl-1016C
dl-R1-747C
dl-R1-445C
dl-R1-332C
dl-R1-319C
dl-R1-309C
dl-R1-201C
dl-R1-145C
dl-R1-93C
Gal4-R1
Gal4-RBP1dlR1
Gal4-RBP1
RBP1-HA
Gal4 alone
G5TKCAT
120.0
100.0
80.0
60.0
40.0
20.0
0.0
percentage / β-gal

Fig. 8A

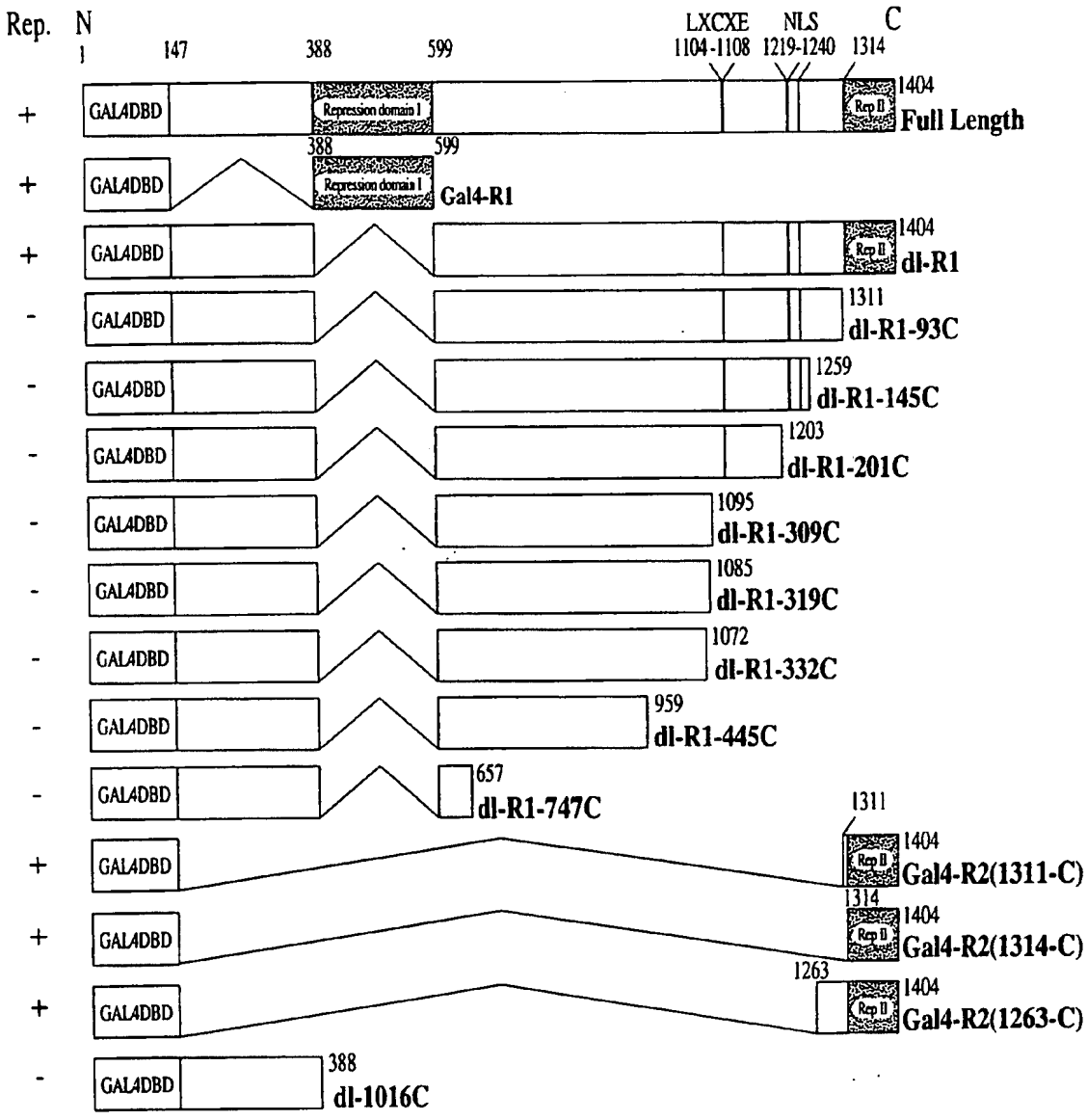

Rep.
N
C
1
147
388
599
LXCXE
1104-1108
NLS
1219-1240
1314
1404
+ GAL4DBD Repression domain I Rep II Full Length
388
599
+ GAL4DBD Repression domain I Gal4-R1
+ GAL4DBD 1404 Rep II dl-R1
- GAL4DBD 1311 dl-R1-93C
- GAL4DBD 1259 dl-R1-145C
- GAL4DBD 1203 dl-R1-201C
- GAL4DBD 1095 dl-R1-309C
- GAL4DBD 1085 dl-R1-319C
- GAL4DBD 1072 dl-R1-332C
- GAL4DBD 959 dl-R1-445C
- GAL4DBD 657 dl-R1-747C
1311
+ GAL4DBD 1404 Rep II Gal4-R2(1311-C)
1314
+ GAL4DBD 1404 Rep II Gal4-R2(1314-C)
1263
+ GAL4DBD 1404 Rep II Gal4-R2(1263-C)
- GAL4DBD 388 dl-1016C

Fig. 8B

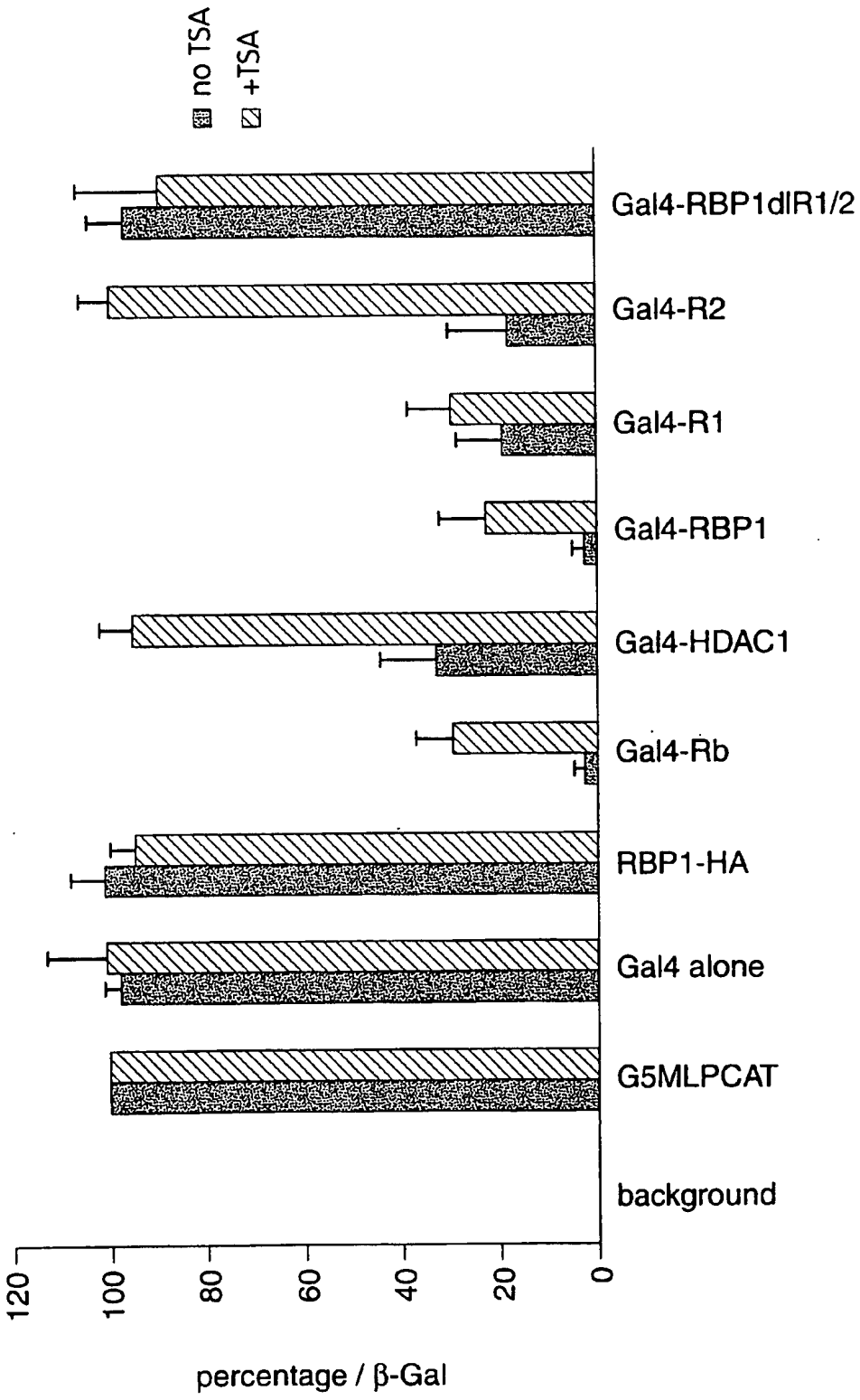

no TSA
+TSA
Gal4-RBP1dlR1/2
Gal4-R2
Gal4-R1
Gal4-RBP1
Gal4-HDAC1
Gal4-Rb
RBP1-HA
Gal4 alone
G5MLPCAT
background
120
100
80
60
40
20
0
percentage / β-Gal

Fig. 8C

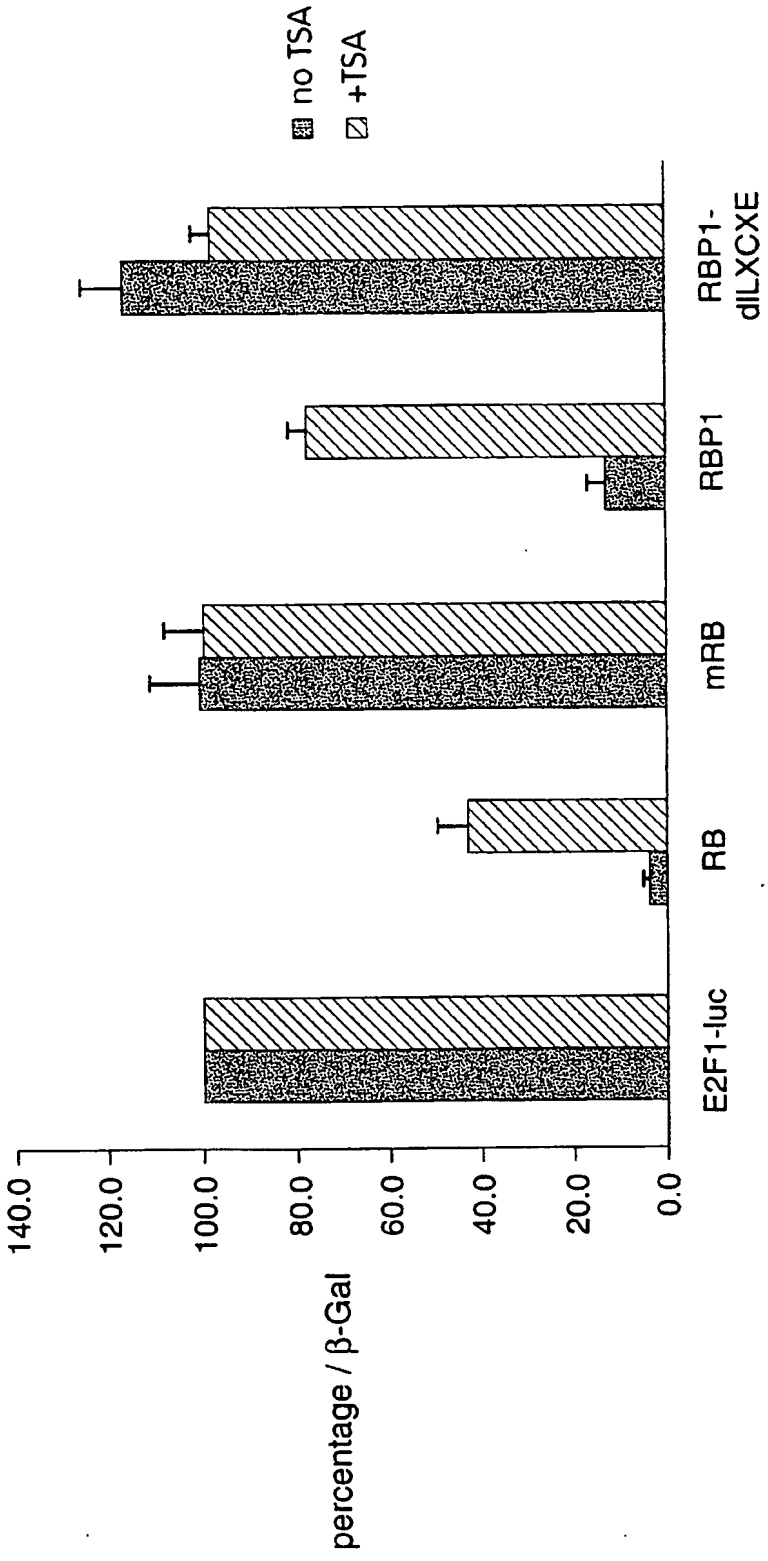

no TSA
+TSA
percentage / β-Gal
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
E2F1-luc
RB
mRB
RBP1
RBP1-dILXCXE

Fig. 8D

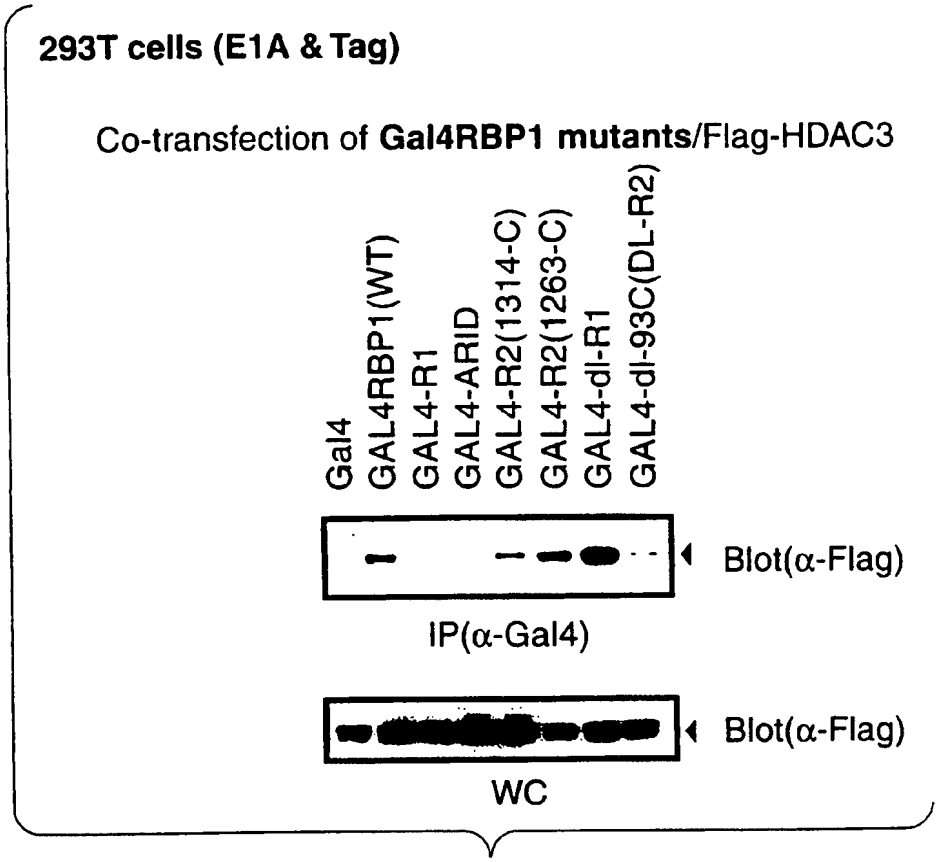

293T cells (E1A & Tag)
Co-transfection of Gal4RBP1 mutants/Flag-HDAC3
Gal4
GAL4RBP1(WT)
GAL4-R1
GAL4-ARID
GAL4-R2(1314-C)
GAL4-R2(1263-C)
GAL4-dl-R1
GAL4-dl-93C(DL-R2)
Blot(α-Flag)
IP(α-Gal4)
Blot(α-Flag)
WC

Fig. 9

SEQUENCE LISTING

<110> GeminX, Inc.

<120> RBP1 POLYPEPTIDES AND USES THEREOF

<130> 50013/018WO2

<150> 60/143,315
<151> 1999-07-12

<160> 4

<170> FastSEQ for Windows Version 3.0

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<211> 26
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<213> Artificial Sequence

<220>
<223> Synthetic based on Homo sapiens

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<210> 2
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic based on Homo sapiens

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1 5 10 15

<210> 3
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic based on Homo sapiens

<400> 3
Asn Glu Phe Tyr Asp Gly Asp His Asp Asn Asp Lys Glu Ser Asp Val
1 5 10 15
Glu Ile

<210> 4

<211> 1257
<212> PRT
<213> Homo sapiens

<400> 4

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 1               5                  10                  15
Ser Ala Lys Tyr Arg Gly Ala Phe Cys Glu Ala Lys Ile Lys Thr Val
            20                  25                  30
Lys Arg Leu Val Lys Val Lys Val Leu Leu Lys Gln Asp Asn Thr Thr
        35                  40                  45
Gln Leu Val Gln Asp Asp Gln Val Lys Gly Pro Leu Arg Val Gly Ala
    50                  55                  60
Ile Val Glu Thr Arg Thr Ser Asp Gly Ser Phe Gln Glu Ala Ile Ile
65                  70                  75                  80
Ser Lys Leu Thr Asp Ala Ser Trp Tyr Thr Val Val Phe Asp Asp Gly
                85                  90                  95
Asp Glu Arg Thr Leu Arg Arg Thr Ser Leu Cys Leu Lys Gly Glu Arg
            100                 105                 110
His Phe Ala Glu Ser Glu Thr Leu Asp Gln Leu Pro Leu Thr Asn Pro
        115                 120                 125
Glu His Phe Gly Thr Pro Val Ile Ala Lys Lys Thr Asn Arg Gly Arg
    130                 135                 140
Arg Ser Ser Leu Pro Val Thr Glu Asp Glu Lys Glu Glu Glu Ser Ser
145                 150                 155                 160
Glu Glu Glu Asp Glu Asp Lys Arg Arg Leu Asn Asp Glu Leu Leu Gly
                165                 170                 175
Lys Val Val Ser Val Val Ser Ala Thr Glu Arg Thr Glu Trp Tyr Pro
            180                 185                 190
Ala Leu Val Ile Ser Pro Ser Cys Asn Asp Asp Ile Thr Val Lys Lys
        195                 200                 205
Asp Gln Cys Leu Val Arg Ser Phe Ile Asp Ser Lys Phe Tyr Ser Ile
    210                 215                 220
Ala Arg Lys Asp Ile Lys Glu Val Asp Ile Leu Asn Leu Pro Glu Ser
225                 230                 235                 240
Glu Leu Ser Thr Lys Pro Gly Leu Gln Lys Ala Ser Ile Phe Leu Lys
                245                 250                 255
Thr Arg Val Val Pro Asp Asn Trp Lys Met Asp Ile Ser Glu Ile Leu
            260                 265                 270
Glu Ser Ser Ser Ser Asp Asp Glu Asp Gly Pro Ala Glu Glu Asn Asp
        275                 280                 285
Glu Glu Lys Glu Lys Glu Ala Lys Lys Thr Glu Glu Glu Val Pro Glu
    290                 295                 300
Glu Glu Leu Asp Pro Glu Glu Arg Asp Asn Phe Leu Gln Gln Leu Tyr
305                 310                 315                 320
Lys Phe Met Glu Asp Arg Gly Thr Pro Ile Asn Lys Pro Pro Val Leu
                325                 330                 335
Gly Tyr Lys Asp Leu Asn Leu Phe Lys Leu Phe Arg Leu Val Tyr His
            340                 345                 350
Gln Gly Gly Cys Asp Asn Ile Asp Ser Gly Ala Val Trp Lys Gln Ile
        355                 360                 365
Tyr Met Asp Leu Gly Ile Pro Ile Leu Asn Ser Ala Ala Ser Tyr Asn
    370                 375                 380
Leu Lys Thr Ala Tyr Arg Lys Tyr Leu Tyr Gly Phe Glu Glu Tyr Cys
385                 390                 395                 400
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Arg Ser Ala Asn Ile Gln Phe Arg Thr Val His His His Glu Pro Lys
405 410 415
Val Lys Glu Glu Lys Lys Asp Leu Glu Glu Ser Met Glu Glu Ala Leu
420 425 430
Lys Leu Asp Gln Glu Met Pro Leu Thr Glu Val Lys Ser Glu Pro Glu
435 440 445
Glu Asn Ile Asp Ser Asn Ser Glu Ser Glu Arg Glu Glu Ile Glu Leu
450 455 460
Lys Ser Pro Arg Gly Arg Arg Arg Ile Ala Arg Asp Val Asn Ser Ile
465 470 475 480
Lys Lys Glu Ile Glu Glu Glu Lys Thr Glu Asp Lys Leu Lys Asp Asn
485 490 495
Asp Thr Glu Asn Lys Asp Val Asp Asp Asp Tyr Glu Thr Ala Glu Lys
500 505 510
Lys Glu Asn Glu Leu Leu Leu Gly Arg Lys Asn Thr Pro Lys Gln Lys
515 520 525
Glu Lys Lys Ile Lys Lys Gln Glu Asp Ser Asp Lys Asp Ser Asp Glu
530 535 540
Glu Glu Glu Lys Ser Gln Glu Arg Glu Glu Thr Glu Ser Lys Cys Asp
545 550 555 560
Ser Glu Gly Glu Glu Asp Glu Glu Asp Met Glu Pro Cys Leu Thr Gly
565 570 575
Thr Lys Val Lys Val Lys Tyr Gly Arg Gly Lys Thr Gln Lys Ile Tyr
580 585 590
Glu Ala Ser Ile Lys Ser Thr Glu Ile Asp Asp Gly Glu Val Leu Tyr
595 600 605
Leu Val His Tyr Tyr Gly Trp Asn Val Ser Tyr Asp Glu Trp Val Lys
610 615 620
Ala Asp Arg Ile Ile Trp Pro Leu Asp Lys Gly Gly Pro Lys Lys Lys
625 630 635 640
Gln Lys Lys Lys Ala Lys Asn Lys Glu Asp Ser Glu Lys Asp Glu Lys
645 650 655
Arg Asp Glu Glu Arg Gln Lys Ser Lys Arg Gly Arg Pro Pro Leu Lys
660 665 670
Ser Thr Leu Ser Ser Asn Met Pro Tyr Gly Leu Ser Lys Thr Ala Asn
675 680 685
Ser Glu Gly Lys Ser Asp Ser Cys Ser Ser Asp Ser Glu Thr Glu Asp
690 695 700
Ala Leu Glu Lys Asn Leu Ile Asn Glu Glu Leu Ser Leu Lys Asp Glu
705 710 715 720
Leu Glu Lys Asn Glu Asn Leu Asn Asp Asp Lys Leu Asp Glu Glu Asn
725 730 735
Pro Lys Ile Ser Ala His Ile Leu Lys Glu Asn Asp Arg Thr Gln Met
740 745 750
Gln Pro Leu Glu Thr Leu Lys Leu Glu Val Gly Glu Asn Glu Gln Ile
755 760 765
Val Gln Ile Phe Gly Asn Lys Met Glu Lys Ala Glu Glu Val Lys Lys
770 775 780
Glu Ala Glu Lys Ser Pro Lys Gly Lys Gly Arg Arg Ser Lys Thr Lys
785 790 795 800
Asp Leu Ser Leu Glu Ile Ile Lys Ile Ser Ser Phe Gly Gln Asn Glu
805 810 815
Ala Gly Ser Glu Pro His Ile Glu Ala His Ser Leu Glu Leu Ser Ser
820 825 830
Leu Asp Asn Lys Asn Phe Ser Ser Ala Thr Glu Asp Glu Ile Asp Gln

835 840 845
Cys Val Lys Glu Lys Lys Leu Lys Arg Lys Ile Leu Gly Gln Ser Ser
850 855 860
Pro Glu Lys Lys Ile Arg Ile Glu Asn Gly Met Glu Met Thr Asn Thr
865 870 875 880
Val Ser Gln Glu Arg Thr Ser Asp Cys Ile Gly Ser Glu Gly Met Lys
885 890 895
Asn Leu Asn Phe Glu Gln His Phe Glu Arg Glu Asn Glu Gly Met Pro
900 905 910
Ser Leu Ile Ala Glu Ser Asn Gln Cys Ile Gln Gln Leu Thr Ser Glu
915 920 925
Arg Phe Asp Ser Pro Ala Glu Glu Thr Val Asn Ile Pro Leu Lys Glu
930 935 940
Asp Glu Asp Ala Met Pro Leu Ile Gly Pro Glu Thr Leu Val Cys His
945 950 955 960
Glu Val Asp Leu Asp Asp Leu Asp Glu Lys Asp Lys Thr Ser Ile Glu
965 970 975
Asp Val Ala Val Glu Ser Ser Glu Ser Asn Ser Leu Val Ser Ile Pro
980 985 990
Pro Ala Leu Pro Pro Val Val Gln His Asn Phe Ser Val Ala Ser Pro
995 1000 1005
Leu Thr Leu Ser Gln Asp Glu Ser Arg Ser Val Lys Ser Glu Ser Asp
1010 1015 1020
Ile Thr Ile Glu Val Asp Ser Ile Ala Glu Glu Ser Gln Glu Gly Leu
1025 1030 1035 104
Cys Glu Arg Glu Ser Ala Asn Gly Phe Glu Thr Asn Val Ala Ser Gly
1045 1050 1055
Thr Cys Ser Ile Ile Val Gln Glu Arg Glu Ser Arg Glu Lys Gly Gln
1060 1065 1070
Lys Arg Pro Ser Asp Gly Asn Ser Gly Leu Met Ala Lys Lys Gln Lys
1075 1080 1085
Arg Thr Pro Lys Arg Thr Ser Ala Ala Ala Lys Asn Glu Lys Asn Gly
1090 1095 1100
Thr Gly Gln Ser Ser Asp Ser Glu Asp Leu Pro Val Leu Asp Asn Ser
1105 1110 1115 112
Ser Lys Cys Thr Pro Val Lys His Leu Asn Val Ser Lys Pro Gln Lys
1125 1130 1135
Leu Ala Arg Ser Pro Ala Arg Ile Ser Pro His Ile Lys Asp Gly Glu
1140 1145 1150
Lys Asp Lys His Arg Glu Lys His Pro Asn Ser Ser Pro Arg Thr Tyr
1155 1160 1165
Lys Trp Ser Phe Gln Leu Asn Glu Leu Asp Asn Met Asn Ser Thr Glu
1170 1175 1180
Arg Ile Ser Phe Leu Gln Glu Lys Leu Gln Glu Ile Arg Lys Tyr Tyr
1185 1190 1195 120
Met Ser Leu Lys Ser Glu Val Ala Thr Ile Asp Arg Arg Arg Lys Arg
1205 1210 1215
Leu Lys Lys Lys Asp Arg Glu Val Ser His Ala Gly Ala Ser Met Ser
1220 1225 1230
Ser Ala Ser Ser Asp Thr Gly Met Ser Pro Ser Ser Ser Ser Pro Pro
1235 1240 1245
Gln Asn Val Leu Ala Val Glu Cys Arg
1250 1255

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/IB 99/01690

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/47 C07K16/18 A61K38/17 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALI R FATTAEY ET AL: "Characterization of the retinoblastoma binding proteins RBP1 and RBP2" ONCOGENE,GB,BASINGSTOKE, HANTS, vol. 8, no. 11, November 1993 (1993-11), pages 3149-3156-3156, XP002107565 ISSN: 0950-9232 cited in the application the whole document	1-22
X	DEFEO-JONES, DEBORAH ET AL: "Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product" NATURE (LONDON), 1991, 352, 251-4, XP000872842 cited in the application the whole document	1-22

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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 2000	24/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hillenbrand, G

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INTERNATIONAL SEARCH REPORT

Intl. Application No
PCT/IB 99/01690

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OTTERSON GA ET AL: "Alternative splicing of the *RBP1* gene clusters in an internal exon that encodes potential phosphorylation sites." ONCOGENE, APR 1993, 8 (4) P949-57, XP000872340 ENGLAND cited in the application the whole document	1-22
X	LAI A ET AL: "*RBP1* induces growth arrest by repression of E2F-dependent transcription." ONCOGENE, MAR 25 1999, 18 (12) P2091-100, XP000872491 ENGLAND the whole document	1-22
A	WO 93 15227 A (UNIV DUKE) 5 August 1993 (1993-08-05) the whole document	1-22

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCT/IB 99/01690

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9315227 A	05-08-1993	AU 3609693 A	01-09-1993
		US 5650287 A	22-07-1997